

922 U.S. PTO

FORM PTO-1390
(REV 12-29-99)

U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

PTO/PCT Rec'd 31 AUG 2000

ATTORNEY'S DOCKET NUMBER

TRANSMITTAL LETTER TO THE UNITED STATES
DESIGNATED/ELECTED OFFICE (DO/EO/US)
CONCERNING A FILING UNDER 35 U.S.C. 371

98-1895

U.S. APPLICATION NO. (If known, see 37 CFR 1.5)

09/623568

INTERNATIONAL APPLICATION NO.

PCT/US99/04996

INTERNATIONAL FILING DATE

05 MARCH 1999

PRIORITY DATE CLAIMED

05 MARCH 1998

TITLE OF INVENTION USE OF A HUMAN HOMOLOG OF A NUCLEAR MIGRATION GENE FOR
TREATMENT AND DIAGNOSIS OF CANCER

APPLICANT(S) FOR DO/EO/US Barbara Miller, Stephen Osmani, Gary Clawson,
Min-Ying Zhang and James Norris

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C.371(b) and PCT Articles 22 and 39(1).
4. A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. A copy of the International Application as filed (35 U.S.C. 371(c)(2))
 - a. is transmitted herewith (required only if not transmitted by the International Bureau).
 - b. has been transmitted by the International Bureau.
 - c. is not required, as the application was filed in the United States Receiving Office (RO/US).
6. A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. Amendments to the claims of the International Application under PCT Article 19(35 U.S.C. 371(c)(3))
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 - b. have been transmitted by the International Bureau.
 - c. have not been made; however, the time limit for making such amendments has NOT expired.
 - d. have not been made and will not be made.
8. A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern document(s) or information included:

11. An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. A **FIRST** preliminary amendment.
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14. A substitute specification.
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Applicant, Patentee, or Identifier: <u>B. MILLER, et al.</u>	
Application or Patent No.: _____	
Filed or Issued: <u>August 31, 2000</u>	
Title: <u>USE OF HUMAN HOMOLOG OF A NUCLEAR MIGRATION GENE FOR TREATMENT AND DIAGNOSIS OF CANCER</u>	
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HUMAN HOMOLOG OF A NUCLEAR MIGRATION AND ITS USE

5

BACKGROUND OF THE INVENTION

This work was supported by National Institutes of Health Grants R01 DK 46778, M01 RR10732, and R01 GM 42564.

10 This invention relates use of a human homolog of a nuclear migration gene in *Aspergillus* for the treatment and diagnosis of human cancer and other diseases and conditions of uncontrolled cell growth.

Nuclear migration is a prominent feature of fundamental biological processes including separation of daughter nuclei during mitosis, fusion of pronuclei during
15 fertilization, and interphase nuclear positioning. Although nuclear movement is of fundamental importance in development of eukaryotes, little is known about the proteins which regulate this process. The filamentous fungus *Aspergillus nidulans nudC* gene has an essential function in movement of nuclei following mitosis and is required for normal colony growth. The identification of heat-sensitive (ts-) *nud* (for *nuclear distribution*)
20 mutants in *Aspergillus nidulans* that prevent nuclear migration into the mycelium demonstrates that nuclear migration is an active process (Xiang *et al.*, 1995). In *A. nidulans*, five nuclear migration genes have been identified. Mutant nuclei divide normally but remain clumped in the spore end of the germ tube.

The gene products of four of the *nud* genes, *nudA*, *nudC*, *nudF*, and *nudG* have
25 been identified after their cloning by complementation of the ts- phenotype using genomic library DNA and sequence analysis. *NudA* encodes a cytoplasmic dynein heavy chain and *nudG* encodes a dynein light chain. Cytoplasmic dynein, in association with microtubules, appears to provide the motor for post-mitotic nuclear movement and is also implicated in vesicle movement and mitosis in animal cells. *NudF* encodes a regulator
30 of an unknown aspect of dynein motor function. *NudF* has 42% sequence identity to the human *LIS-1* (Miller-Dieker lissencephaly-1) gene, which is required for proper neuronal migration during brain development and is also a subunit of intracellular platelet-

-2-

activating factor acetylhydrolase (Xiang et al., 1995; Reiner et al., 1993). The *nudC* gene product in *A. nidulans* may interact between microtubules and nuclei and is involved in nuclear movement (Osmani et al., 1990). The precise function is not known, but NUDC is required to maintain levels of NUDF, and *nudF* was first identified as a copy number
5 suppressor of the *nudC3* mutation. Data on genetic and biochemical interactions in *A. nidulans* suggest that NUDC acts upstream of NUDF, which then interacts with the NUDA dynein motor protein to mediate controlled nuclear migration. Consistent with this genetic interaction, LIS-1 and NUDC have recently been found to physically interact, although these proteins do not appear to associate in *A. nidulans*. In *A. nidulans*, the
10 effect of the *nudC3* mutation is to inhibit nuclear migration and to reduce colony growth. Although deletion of *nudA* and NUDF affects nuclear migration, deletion of *nudC* resulted in a more severe phenotype profoundly affecting morphology and composition of the cell wall and resulting in lethality (Chiu et al., 1997). Spores of strains deleted for *nudC* grow spherically and lyse. The thickness of the cell wall is increased in the
15 deletion mutant, and wall polymer composition and actin distribution are abnormal. These data demonstrate that in *A. nidulans nudC*, unlike *nudA* and *nudF*, plays a significant role in cell wall morphogenesis and cell growth in addition to nuclear migration. Thus, in *A. nidulans*, NUD proteins have a significant role in both nuclear migration and cell growth.

20 Homologs of the *A. nidulans nudC* gene have been identified in *Drosophila* (Cunniff et al., 1997) and a mammalian *nudC* gene has recently been cloned in rat lymphoma cells. (Axtell et al., 1995; Yu-Lee et al., 1990) This gene, c15, was one of 26 prolactin-responsive cDNA's isolated by differential screening of a λ ZAP Nb2 T cell cDNA library. The 1.7 kb mRNA contains one large open reading frame and encodes a
25 protein of 332 amino acids (45 kDa). The amino terminus of c15, which does not overlap with the smaller *A. nidulans* protein (22 kDa) contains a basic stretch similar to the nuclear localization signal found in proteins which may be involved in protein/protein interactions. Stimulation of Nb2 T rat lymphoma cells with prolactin or interleukin-2 enhanced the expression of *nudC* mRNA, which peaked in 8-10 hours, at the G1/S
30 transition of mitosis.

Information on human nuclear migration genes is needed to determine whether these genes affect human diseases, e.g., cancers, and, if so, what manipulations of the genes or gene product may be used for treatment or diagnosis.

SUMMARY OF THE INVENTION

5 The human homolog of an *Aspergillus* nuclear migration gene, the *nudC* gene, was cloned and sequenced. The amino acid sequence of the rat and human NUDC proteins are 94% identical. The amino terminus of the larger human protein (HNUDC = 45 kDa) does not overlap with *A. nidulans* NUDC (22 kDa). However, NUDC and the c-terminal 94 amino acids of HNUDC are 67% identical. The C-terminal region of the
10 *HnudC* gene fully complemented the *A. nidulans* temperature-sensitive *nudC3* mutation and restored nuclear migration and colony growth in a temperature-sensitive mutant, suggesting that the *A. nidulans* and human *nudC* genes have a related role in cell proliferation. The ability of *Drosophila* and rat *nudC* to also complement the *A. nidulans* temperature-sensitive *nudC3* mutation function suggests that *nudC* has an essential
15 function in cell growth that is conserved from filamentous fungi to humans.

A twelve amino acid sequence was identified in the carboxy terminus of the NUDC protein which is conserved in *A. nidulans*, *Drosophila*, rat and human NUDC proteins. Antibodies were generated to this conserved sequence and to a fifteen amino acid sequence specific for the human NUDC protein. Although significant quantities of
20 *HnudC* mRNA were found in all tissues examined, HNUDC protein was differentially expressed, with large amounts found in two divergent tissues, human brain, and progenitor-derived hematopoietic precursors. Because high expression of *HnudC* was observed in normal erythroid precursors compared to other tissues, the expression and function of *nudC* in human hematopoietic proliferation and differentiation were
25 examined. In normal human bone marrow, HNUDC protein has a dynamic expression pattern, being highly expressed in early hematopoietic myeloid and erythroid precursors and declining during differentiation. Expression of *nudC* in human hematopoietic proliferation and differentiation was examined. Expression of HNUDC protein and mRNA was greatest in normal proliferating erythroid precursors and declined as these
30 cells terminally differentiated. Nuclear/cytoplasmic fractionation demonstrated that in these cells, HNUDC is primarily a cytoplasmic protein, and immunohistochemistry demonstrated the localization to be primarily paranuclear.

To determine whether hematopoietic growth factors induce *HnudC* expression, TF-1 cells, a growth factor-dependent human erythroleukemia cell line, were stimulated by GM-CSF. This induced a significant increase in HNUDC protein and in *HnudC* mRNA, suggesting that enhancement of *HnudC* expression in response to growth factor stimulation may be mediated at the level of transcription. The inducible expression of *HnudC* mRNA and protein demonstrated here in response to GM-CSF suggests that the conserved product of this newly identified gene has an important function in the proliferation of human hematopoietic cells. HNUDC was also highly expressed in all hematopoietic cell lines examined.

The ability of human *nudC* to restore normal nuclear migration and colony growth to an *A. nidulans* mutant and its inducible expression in response to hematopoietic growth factor stimulation indicate that *nudC* has an important function in mammalian cell growth which is conserved in hematopoietic cells.

Functional experiments performed on TF-1 cells treated with phosphorothioate antisense oligonucleotides to *HnudC* mRNA demonstrated a significant dose-dependent reduction in TF-1 proliferation in anti-*HnudC* oligomer-treated cells, providing evidence that HNUDC is an essential protein in human hematopoietic cell growth.

HNUDC expression was also significantly enhanced in bone marrow aspirates from patients with acute myelogenous (AML) and acute lymphoblastic (ALL) leukemia compared to aspirates from normal controls, suggesting that *HnudC* is involved in malignant hematopoietic cell growth as well. These data demonstrate that HNUDC is a conserved protein highly expressed in normal and malignant human hematopoietic precursors and of functional importance in hematopoietic cell proliferation.

Marked up regulation of *HnudC* expression was demonstrated in the lysates of bone marrow aspirates from patients with acute myelogenous (AML) and acute lymphoblastic (ALL) leukemia, strongly suggesting that HNUDC is involved in malignant hematopoietic proliferation. Retrospective analysis of pediatric clinical trials have established prognostic factors that subsequently have been applied prospectively to stratify patients into different treatment groups according to their relative risk of relapse. This has resulted in the classification of childhood ALL patients as "standard" or "high risk." Higher levels of *HnudC* were observed in the aspirates of high risk and relapsed pediatric ALL patients compared to standard risk patients. Therefore, a diagnostic use

of measurement of HNUDC expression is to identify a patient population at higher risk of relapse, who requires more intensive therapy to eliminate the malignant cell population. High expression of *HnudC* may be a marker of a more aggressive phenotype for other malignancies as well. Because deletion of *nudC* results in lethality in *A. nidulans* and antisense studies significantly reduced the proliferation, targeted down regulation of HNUDC expression is a therapeutic option to control the proliferation of leukemia cells. Use of targeted down regulation of HNUDC to control the growth of malignant tumors which express HNUDC including leukemias, is an aspect of the invention.

10 Antisense oligonucleotides to *HnudC* are a therapeutic option, however may have a number of limitations including instability and non-specific toxicity. RNAs which are abundant, or have significant secondary structure are unlikely to be modulated efficiently. Ribozymes are an alternative approach to down regulating specific genes. Ribozymes are catalytic RNA molecules that recognize their target RNA in a highly sequence-specific manner. Ribozyme-mediated cleavage occurs 3' to a targeted nucleotide triplet NUX (N can be any nucleotide, whereas X can be A, C or U). Specificity is conferred by flanking sequences which extend on both sides of the target site, and even relatively short flanking sequences (6-9 nucleotides each) allow both sufficient specificity for the cleavage reaction as well as ready dissociation from the target, which is rate-limiting.

15 Triple ribozyme (TRz) constructs are an aspect of the invention which have distinct advantages. These TRz consist of two cis-acting ribozymes flanking an internal trans-acting ribozyme, which is targeted to a cellular RNA. The activity of the two cis-acting ribozymes efficiently liberates the internal ribozyme with minimal non-specific flanking sequences. The liberated internal ribozyme is 3-8 times more active than the same

20 ribozyme within non-specific flanking sequences, and self-liberation provides a distribution of active internal ribozyme between nucleus and cytoplasm.

TRz is useful to inhibit *HnudC* mRNA expression. To prepare and target ribozymes to *HnudC*, the basic reagent encompasses a TRz whose expression is controlled by an inducible or tissue-specific promoter preferably in a vector. Examples

30 of inducers are ectosomal or tetracycline driven. Tissue specific promoters include a CD19 promoter for ALL, for example. CD19 is an antigen expressed in the majority of B cell ALL patients, that is, most pediatric cases. Triple ribozymes targeted to *HnudC*

mRNA were prepared. These ribozymes have been library selected and are very active against *HnudC* mRNA *in vitro* (Lieber and Strauss, 1995). *HnudC* levels were modulated using the *HnudC* triple ribozymes and the *HnudC* TRz was used to study function by modulating expression. Their inducible expression in hematopoietic cell cultures revealed how lack of HNUDC affects cell proliferation, cell survival, and cell cycle progression. *HnudC* levels were modulated using the *HnudC* triple ribozymes and the *HnudC* TRz was used to study function by modulating expression.

In summary, the nuclear migration gene *HnudC* and its gene products open a vista of possibilities to control abnormal human cell proliferation such as malignant growth. Means for control that offer treatment strategies include antisense molecules and triple ribozymes. Oligonucleotide probes and antibodies to HNUDC are useful for diagnosis and clinical staging of cancers in particular, leukemias.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 presents an amino acid sequence analysis of *HnudC* comparing human (HUM), rat, and *A. nidulans* (ASP) *nudC* homologs; differences between the rat and human proteins are indicated in ***bold italics***; the region of 12 amino acids perfectly conserved between all three is indicated by double underlines (using the well-known single letter code for amino acids).

FIG. 2 demonstrates complementation of the *A. nidulans nudC3* mutation by *HnudC*; similar complementation was obtained with either *A. nidulans nudC* cDNA or its human counterpart *HnudC* cloned into the pAL5 expression vector; no complementation was observed after transformation using the empty pAL5 vector.

FIG. 3 illustrates the specificity of anti-peptide antibody to NUDC:

(A) antibody was prepared to a 12 amino acid peptide conserved in *A. nidulans*, *Drosophila*, rat, and human NUDC. *A. nidulans* extract from cells uninduced (UI) or induced (I) to over express NUDC, along with protein from day 10 BFU-E derived human erythroblasts, were loaded onto a 10% polyacrylamide gel; blots were developed with preimmune sera, anti-CT peptide immune sera, or affinity purified anti-CT peptide antibody;

(B) antibody was also prepared to a 15 amino acid peptide specific for human NUDC; 5, 20, or 50 μ g of protein extract from TF-1 cells was loaded in each lane of a 10% polyacrylamide gel; blots were developed with preimmune sera, affinity purified

anti-MID peptide antibody, or affinity purified anti-MID antibody incubated prior to Western blotting with MID NUDC peptide.

FIG. 4 shows the immunohistochemistry of HNUDC in a normal bone marrow biopsy prepared with anti-MID HNUDC antibody (A) or preimmune sera (B).

5 FIG. 5 shows the immunohistochemistry of HNUDC in BFU-E derived cells; cytochrome preparations of day 7 (A,D), 10 (B,E), and 14 (C,F) BFU-E derived cells were prepared with anti-CT NUDC peptide antibody (A,B,C) or preimmune sera (D,E,F).

FIG. 6 illustrates *HnudC* expression in day 7, 10, and 14 BFU-E derived erythroblasts; (A) shows a Western blot; (B) shows results of RT-PCR; and (C) shows a Western blot of HNUDC in nuclear and cytoplasmic fractions of day 10 cells.

FIG. 7 presents stimulation of *HnudC* expression in TF-1 cells by GM-CSF:

(A) shows a Western blot of lysates of TF-1 cells stimulated with GM-CSF for 0-24 hours; detection was with anti-CT NUDC peptide antibody and ECL.

(B) shows a Northern blot analysis of *HnudC* mRNA from TF-1 cells stimulated for 0-24 hours with GM-CSF; 18S rRNA is the control.

FIG. 8 presents the effect of antisense oligonucleotides on TF-1 cell growth, TF-1 cells were transfected with lipofectin, or with 2.5 or 5 μ g/ml antisense or sense oligonucleotides targeted to *HnudC* mRNA; *indicates a significant decrease.

FIG. 9 shows a Western blot of HNUDC in bone marrow aspirates of normal donors, patients with ALL and AML, and TF-1 cells; equivalent amounts of protein were loaded.

FIG. 10 is an *in vitro* demonstration of efficient cleavage of *HnudC* mRNA by the anti-*HnudC* target sequences identified by the library selection method; ribozymes are numbered by the *HnudC* sequence they recognize consisting of one (2,3,4,6) or combinations of two internal ribozymes; the control ribozyme had no anti-*HnudC* sequence inserted.

FIG. 11 shows the nucleotide sequence of the *HnudC* cDNA.

DETAILED DESCRIPTION OF THE INVENTION

A human homolog of a nuclear migration gene in *Aspergillus* was cloned and sequenced. Antibodies were developed to a fragment of the human protein which were used to investigate distribution and function of the protein, and to interfere with the protein, and to interfere with the protein's function. Antisense oligonucleotides were

developed that interfere with *HnudC* expression. Triple ribozymes can also be used for such interference. Oligonucleotide proteins are useful for diagnosing the presence of the gene or the levels of mRNA.

Cloning and Sequence Analysis of Human *NudC*.

5 Because of the essential role of *nudC* in nuclear migration and in colony growth in *A. nidulans*, an EST Human cDNA Database (TIGR) was searched with the *A. nidulans nudC* sequence to identify potential human homologs, which could be relevant in human diseases involving cell growth. Two cDNA clones were identified which had a high degree of homology and these were obtained from the ATCC. Full sequencing of
10 these two clones revealed a high degree of sequence identity to the carboxy terminus of rat and *A. nidulans nudC*, but the amino terminus of the larger rat *nudC* gene was missing from both human clones.

RACE PCR was used to determine the complete human *nudC* gene sequence. First, a Multi-tissue Northern Blot (Clontech) was obtained and probed with random
15 primer labeled partial human *nudC* (designated *HnudC*) cDNA from the 108447 ATCC clone or the β -actin cDNA probe as a control. *HnudC* mRNA was found in all tissue examined. Because significant quantities of *HnudC* mRNA were present in heart, Marathon-Ready Human Heart cDNA was obtained from Clontech (Palo Alto, CA) and 5' and 3'-RACE performed with primers as described herein. Single 5' and 3'-RACE PCR
20 products were obtained and their identity was confirmed with Southern blotting using the labeled ATCC partial *HnudC* sequence as a probe. Sequencing of the 5' and 3'-RACE PCR products revealed the full nucleotide sequence of the *HnudC* gene (Accession #AF130736). A comparison between human, rat, and *A. nidulans* NUDC homologs is shown in FIG. 1. The nucleotide sequence of a cDNA is shown in FIG. 11.

25 FIG. 1 presents an amino acid sequence analysis of *HnudC* comparing human (HUM), rat, and *A. nidulans* (ASP) *nudC* homologs; differences between the rat and human proteins are indicated in ***bold italics***; the region of 12 amino acids perfectly conserved between all three is indicated by double underlines.

The nucleotide sequence of rat and human *nudC* are highly homologous and the
30 encoded amino acid sequence is 94% identical. The amino terminus of human and rat NUDC does not overlap with *A. nidulans* NUDC, which is a smaller protein of 22 kDa (FIG. 1). However, the carboxy terminal 94 amino acids of the human clone are highly

-9-

homologous to rat (98%), *Drosophila* (76%), and *A. nidulans* (67%). Although the amino acid sequences in portions of the carboxy terminal region are similar between the human, rat, *Drosophila* and *A. nidulans* protein, the DNA sequences are different due to a high number of silent mutations which exist. The Prosite Program was used to scan the human NUDC sequence for sites of potential post-translational modifications, targeting and binding domains, DNA or RNA association or enzyme (transferase, hydrolase, or isomerase) activity. Few assignable sites were found. One potential site of cAMP- and cGMP-dependent protein kinase phosphorylation (aa37-40), two of protein kinase C (aa 69-71, 266-268), nine of casein kinase II phosphorylation, one of tyrosine phosphorylation (amino acid 292) and three potential N-myristoylation sites were identified (aa 12-17, 45-50, 300-305).

Complementation of *A. nidulans nudC* Mutants by Human *nudC*.

The ability of *HnudC* to functionally complement the temperature sensitivity of the *nudC3* mutation of *A. nidulans* was determined by transformation. The pAL5 expression vector containing the *alcA* promoter was employed to drive expression of *HnudC* in *A. nidulans*. *A. nidulans nudC* cDNA was used as a positive control and the empty pAL5 vector as a negative control. *nudC* cDNA WAS cloned into pUC18 to obtain complete reversal of the *nudC3* mutation without relying on an heterologous promoter. Transformants were first selected utilizing the *pyr4* nutritional marker on pAL5 or plasmid *pyrG* in the co-transformation with *nudC* cDNA cloned in pUC18. Transformants containing the nutritional marker were selected at a permissive temperature and consequently tested for their ability to grow at the restrictive temperature of 42°C (FIG. 2) using glycerol as the carbon source to allow expression from the *alcA* promoter of the various genes.

FIG. 2 demonstrates complementation of the *A. nidulans nudC3* mutation by *HnudC*; similar complementation was obtained with either *A. nidulans nudC* cDNA or its human counterpart *HnudC* cloned into the pAL5 expression vector; no complementation was observed after transformation using the empty pAL5 vector.

Previous studies reported that cDNA of *A. nidulans* genes can completely complement, presumably by gene conversion, temperature sensitive mutations within that gene. This is shown for *nudC3* (FIG. 2) with nine transformants co-transformed with *pyrG* and *nudC* cDNA in pUC18 showing complete complementation of the ts-

-10-

phenotype when grown at 42°C. Similar complementation was observed for transformants receiving either *A. nidulans nudC* cDNA or human *HnudC* cDNA cloned into pAL5 whereas the empty pAL5 vector failed to complement. All transformants receiving either pAL5 driven *nudC* or *HnudC* were complemented in a similar fashion (FIG. 2). These data demonstrate that human *HnudC* encodes a functional homolog of *A. nidulans nudC*. Complementation of *nudC3* using the *Drosophila* and rat *nudC* like genes was reported by Cunniff *et al.*, 1997 and by Morris *et al.*, 1997, further indicating the high level of conservation of *nudC* function.

Specificity of Anti-Peptide Antibody to NUDC.

To produce antibody with a high degree of specificity for NUDC, a peptide was prepared to a 12 amino acid sequence in the carboxy terminus of NUDC conserved in *A. nidulans*, rat, *Drosophila* and human (GC M V E K M M Y D Q R Q K). The antibody (anti-CT NUDC), which recognizes both the *A. nidulans* (22 kDa) and human (45 kDa) NUDC proteins, was affinity purified as described herein. The specificity of the anti-CT antibody was examined with Western blotting of extracts of *A. nidulans* induced to overexpress NUDC and of human BFU-E derived cells. Detection with preimmune serum, immune serum, or affinity purified antibody, followed by ECL, is shown in FIG. 3A.

FIG. 3 illustrates the specificity of anti-peptide antibody to NUDC:

(A) antibody was prepared to a 12 amino acid peptide conserved in *A. nidulans*, *Drosophila*, rat, and human NUDC. *A. nidulans* extract from cells uninduced (UI) or induced (I) to overexpress NUDC, along with protein from day 10 BFU-E derived human erythroblasts, were loaded onto a 10% polyacrylamide gel; blots were developed with preimmune sera, anti-CT peptide immune sera, or affinity purified anti-CT peptide antibody;

(B) antibody was also prepared to a 15 amino acid peptide specific for human NUDC; 5, 20, or 50 ug of protein extract from TF-1 cells was loaded in each lane of a 10% polyacrylamide gel; blots were developed with preimmune sera, affinity purified anti-MID peptide antibody, or affinity purified anti-MID antibody incubated prior to Western blotting with MID NUDC peptide.

To raise a second antibody to NUDC, a peptide was prepared to a 15 amino acid sequence in the middle of the human NUDC protein (N G S L D S P G K Q D T E E D).

The antibody was affinity purified as described herein. The specificity of this antibody (anti-MID NUDC) was examined with Western blotting of extracts from TF-1 (human erythroleukemia cells) and results are shown in FIG. 3B. Incubation of this antibody overnight with the MID peptide completely blocked the ability of this antibody to recognize the NUDC band on Western blots (FIG. 3B), whereas incubation with the CT peptide had no effect. Likewise, when the anti-CT NUDC antibody was incubated overnight with the CT peptide, recognition of NUDC by the antibody was completely inhibited whereas incubation with the MID NUDC peptide had no blocking effect. These results demonstrate the high specificity of affinity purified anti-CT and anti-MID NUDC peptide antibodies.

NudC Expression in Normal Human Hematopoietic Proliferation and Differentiation.

Affinity purified anti-NUDC peptide antibodies were used in immunohistochemistry experiments to examine the cellular distribution of NUDC in human bone marrow. Ten bone marrow biopsies were examined and a representative bone marrow is shown in FIG. 4. Blasts and early precursors, both myeloid and erythroid, were observed to express high levels of NUDC protein, which was primarily cytoplasmic. Punctate nuclear staining was also observed. Little or no NUDC protein was detected in the most differentiated myeloid and erythroid cells.

FIG. 4 shows the immunohistochemistry of HNUDC in a normal bone marrow biopsy prepared with anti-MID HNUDC antibody (A) or preimmune sera (B).

To confirm this dynamic expression pattern, *HnudC* expression was examined in normal human erythroid precursors at several defined stages of proliferation and differentiation. BFU-E derived colonies were removed from culture on day 7, 10 or 14 of maturation. Day 7 BFU-E derived cells are poorly hemoglobinized blasts with a large proliferative capacity, day 10 cells are partially hemoglobinized proerythroblasts or basophilic normoblasts with less proliferative capacity, and day 14 cells are largely terminally differentiating polychromatophilic and orthochromatic normoblasts. Day 7, 10, and 14 BFU-E derived cells were examined by immunohistochemistry to determine the level of expression and subcellular localization of HNUDC in erythroid precursors at three stages of differentiation. Preimmune sera was used as the control. HNUDC was strongly detected in the paranuclear cytoplasm of day 7 erythroid precursors and

decreased dramatically during maturation (FIG. 5), confirming results obtained with normal bone marrow. Faint nuclear foci were also observed in day 7 and 10 cells, but HNUDC protein primarily localized to the cytoplasm. These experiments were repeated three times with similar results.

5 FIG. 5 shows the immunohistochemistry of HNUDC in BFU-E derived cells; cytocentrifuge preparations of day 7 (A,D), 10 (B,E), and 14 (C,F) BFU-E derived cells were prepared with anti-CT NUDC peptide antibody (A,B,C) or preimmune sera (D,E,F).

 FIG. 6 illustrates *HnudC* expression in day 7, 10, and 14 BFU-E derived erythroblasts; (A) shows a Western blot; (B) shows results of RT-PCR; and (C) shows
10 a Western blot of HNUDC in nuclear and cytoplasmic fractions of day 10 cells.

 Western blotting was performed on extracts from day 7, 10 and 14 cells and detection was performed with the anti-NUDC peptide antibody. Antibody to $\text{I}\kappa\beta\alpha$, a protein with uniform expression in these cells, was used as a control. This confirmed that HNUDC (45 kDa) abundance was greatest in early precursors, which are rapidly
15 proliferating, and significantly declined at day 14 as these erythroid cells terminally differentiated (FIG. 6A, $p < 0.03$). *HnudC* mRNA levels were analyzed in these cells by RT-PCR and significantly decreased from day 7 to 14 of differentiation (FIG. 6B, $p < 0.05$). 18S rRNA mRNA levels were measured as a control. Mean \pm SEM densitometry measurements of *HnudC* mRNA normalized with the 18S rRNA band for
20 5 experiments were 0.44 ± 0.08 , 0.24 ± 0.06 and 0.05 ± 0.02 for day 7, 10 and 14 cells respectively. The decrease in *nudC* mRNA noted in erythroid precursors differentiating from day 7 to 14 could account for the reduction in HNUDC protein.

 Day 10 BFU-E derived cells were fractionated into nuclear or cytosolic extracts, and Western blotting performed. A representative results of two experiments is shown
25 in FIG. 6C, confirming that HNUDC is primarily a cytoplasmic protein in BFU-E derived cells. Antibodies to $\text{I}\kappa\beta\alpha$, a cytoplasmic protein, (Dobrzanski *et al.*, 1995) and E47, a transcription factor, (Kadesch, 1993) were used to confirm the quality of fractionation.

GM-CSF Induces *HnudC* Expression in TF-1 Cells.

 To determine whether cytokine stimulation enhances *HnudC* expression in
30 hematopoietic cells, TF-1 cells were removed from growth factor for 24 hours and then stimulated with GM-CSF. TF-1 is a human erythroleukemia cell line which is absolutely dependent on GM-CSF, IL-3, or erythropoietin for growth. (Kitamura *et al.*, 1989)

Stimulation with GM-CSF induced a significant ($p < 0.05$) increase in HNUDC protein at 16-24 hours. The mean increase in cell number at 24 hours, the approximate cell doubling time, was $241 \pm 36\%$. A representative blot demonstrating the kinetics of HNUDC protein induction is shown in FIG. 7A.

5 FIG. 7 presents stimulation of *HnudC* expression in TF-1 cells by GM-CSF:

(A) shows a Western blot of lysates of TF-1 cells stimulated with GM-CSF for 0-24 hours; detection was with anti-CT NUDC peptide antibody and ECL.

(B) shows a Northern blot analysis of *HnudC* mRNA from TF-1 cells stimulated for 0-24 hours with GM-CSF; 18S rRNA is the control.

10 *HnudC* mRNA expression in response to GM-CSF stimulation was also examined. A Northern blot of *HnudC* mRNA and 18S rRNA prepared from TF-1 cells 0-24 hours following GM-CSF stimulation is shown in FIG. 7B. *HnudC* mRNA densitometry measurements normalized with the 18S rRNA band at hours 0, 8, 10, 16, 20, were 0.12, 0.57, 0.77, 1.11 and 0.82 respectively. The increase in *HnudC* mRNA
15 following growth factor stimulation may explain the increased NUDC protein levels observed. High levels of the HNUDC were observed in all hematopoietic cell lines studied including the lymphoblastic leukemia cell lines JM1 and Reh, AML-193, and K562.

In contrast, erythropoietin stimulation of TF-1 cells did not result in a significant
20 increase in HNUDC protein within 24 hours of growth factor stimulation. At 24 hours after erythropoietin stimulation, the mean increase in cell number was $126 \pm 32\%$. This is consistent with previously published results that erythropoietin does not support the growth of TF-1 cells as well as GM-CSF, possibly due to the erythropoietin receptor mutation found in these cells, and suggests that in these cells, the quantity of HNUDC
25 induced in response to growth factor stimulation may be related to the proliferative response.

The expression of HNUDC in day 7, 10, and 14 BFU-E derived erythroblasts was compared to that in unstimulated and stimulated TF-1 cells. The amount of HNUDC in TF-1 cells, which are rapidly dividing, was much greater than that in normal BFU-E
30 derived cells.

Antisense Oligonucleotide Experiments

Functional studies were performed with TF-1 cells using phosphorothioate antisense or sense oligonucleotides targeted to the N-terminus of *HnudC* mRNA.

FIG. 8 presents the effect of antisense oligonucleotides on TF-1 cell growth, TF-1
5 cells were transfected with lipofectin, or with 2.5 or 5 μ g/ml antisense or sense oligonucleotides targeted to *HnudC* mRNA; *indicates a significant decrease.

FIG. 9 shows a Western blot of HNUDC in bone marrow aspirates of normal donors, patients with ALL and AML, and TF-1 cells; equivalent amounts of protein were loaded.

10 Preliminary experiments were done to examine a variety of oligonucleotide treatments, including both time and oligomer and lipofection dose response curves, to determine the most effective treatment conditions. A six hour treatment with lipofectin/5 μ g/ml antisense or sense oligonucleotide appeared optimal (FIG. 8A); these conditions produced little growth inhibition with the sense phosphorothioate oligonucleotide (75 \pm
15 8%) compared to treatment with lipofectin alone. In contrast, antisense oligonucleotide (to the same coding region as sense) inhibited TF-1 cell growth to 33 \pm 6% ($p \leq 0.001$) of lipofectin control (FIG. 8A). This inhibition was dose-dependent and was observed in three separate experiments. A second antisense oligonucleotide to the mid region of *HnudC* also significantly reduced TF-1 growth compared to treatment with lipofectin
20 alone or the sense control ($p \leq 0.001$).

To determine the effect of antisense/sense oligonucleotide treatment on NUDC protein levels, immunoblot analysis was performed on lipofectin alone, sense or antisense treated TF-1 cells. Results are shown in FIG. 8B and are consistent with effects on TF-1 cell growth observed. NUDC protein was markedly reduced in TF-1 cells treated with
25 5 μ g/ml antisense oligonucleotide compared to sense or lipofectin control.

Role for HNUDC in Leukemic Cell Proliferation

To determine whether *HnudC* expression is enhanced in human leukemia, Western blotting with anti-NUDC antibodies was performed on bone marrow aspirates from ten normal donors, seventeen patients with acute lymphoblastic leukemia (ALL),
30 and four patients with acute myelogenous leukemia (AML) at diagnosis or relapse (2 patients) (FIG. 7). The mean densitometry measurement \pm standard error of the mean (SEM), measured in the linear range, for HNUDC from normal donors and patients with

ALL and AML were 0.089 ± 0.023 , 1.78 ± 0.39 and 1.76 ± 0.82 , respectively. ($p < 0.01$). For four patients with ALL studied at diagnosis and remission, the mean densitometry measurement at diagnosis was 1.59 ± 0.37 and at day 28 (remission) was 0.013 ± 0.001 ($p < 0.01$). Immunohistochemistry was performed on four bone marrow biopsies from
5 patients with ALL at diagnosis. Morphologic analysis of these biopsies revealed greater than 90% blast cell types. HNUDC expression was uniformly enhanced in the cytoplasm of most cells and prominent punctate nuclear staining was again seen. This high expression in immature, malignant precursors supports the conclusion that HNUDC is involved in enhanced hematopoietic proliferation. High levels of HNUDC were observed
10 in four other hematopoietic cell lines of leukemic origin including the lymphoblastic leukemia cell lines JM1 and Reh, AML-193, and K562.

Subcellular Localization of HNUDC

The cellular localization of HNUDC was examined by immunofluorescent staining of TF-1 cells using specific affinity-purified antibodies to both the C-terminal and mid-regions of the protein. HNUDC was observed in both the nucleus and the
15 cytoplasm with both antibodies but not with pre-immune sera or antibody incubated with the cognate peptide prior to cell staining. While no specific staining of cytoskeletal elements was observed in the cytoplasm, nuclear staining consisted of discrete foci. Occasionally cells were observed which had little nuclear staining but contained bright
20 foci in the cytoplasm surrounding the DNA. These cells were always in pairs, suggesting that they may be dividing cells and careful examination of cells doubly stained with HNUDC antibodies and tubulin confirmed that these cells were in late anaphase, telophase, or undergoing cytokinesis. This staining of cytoplasmic foci during late anaphase through to cytokinesis was observed with both antibodies and upon fixation in
25 either cold methanol or para-formaldehyde. To determine which subnuclear compartment HNUDC was localized to, double immunofluorescent staining was carried out with the HNUDC antibodies and a panel of antibodies specific for known nuclear proteins. Interphase HNUDC nuclear foci were found to colocalize with the SC-35 protein, which is a component of spliceosomes. SC-35 is an essential splicing factor
30 which also colocalizes with interchromatin granules and perichromatin fibrils. Examination of cells in late anaphase, telephase, or undergoing cytokinesis indicated that the HNUDC foci in these cells also colocalized with SC-35 during these stages of the cell

cycle. Similar colocalization of the nuclear fraction of HNUDC with SC-35 was also observed in a rhabdomyosarcoma cell line. The presence of HNUDC in foci surrounding the DNA of dividing cells suggests that it may be playing a role in cell division/proliferation by controlling nuclear movements at cytokinesis.

5 Preparation and Targeting of Ribozymes to *HnudC*

Inactivation of gene expression by ribozymes is a powerful technique for studying the function of a gene product and has therapeutic potential as well. The effectiveness of the TRz approach in modulating mRNA and protein expression has already been demonstrated. Using TRzs targeted to the retinoblastoma (Rb) gene mRNA or to the C3 and C9 subunits of multicatalytic proteinase, these ribozymes were efficiently liberated and functioned effectively *in vivo* (Benedict et al., 1998). Analogous results were obtained with TRz targeted to repetitive B2 transcripts and to the C3 and C9 subunits of multicatalytic proteinase, where distribution of liberated internal ribozymes between nucleus (up to 20%) and cytoplasm was demonstrated. (Crone et al., 1999; Ren *et al.*, 1998). Use of active TRz constructs in conjunction with tissue-specific promoters also allows selective gene-knockout strategies. For example, probasin promoter-driven expression of PolII-targeted triple ribozyme resulted in highly selective targeting of the prostate in transgenic mice, where a time-dependent destruction of prostate epithelium (including ablation) was found (Voeks et al., 1998).

20 The basic reagent used herein encompasses a TRz whose expression is controlled by an inducer or tissue-specific promoter. The TRz includes an internal trans-acting ribozyme, which can bind to target RNA. The internal ribozyme is flanked by 2 cis-acting ribozymes which function to cut themselves off, liberating the internal ribozyme. The liberated internal ribozyme is 3-8 times more active than the same ribozyme within non-specific flanking sequences, and the self-liberation process provides a distribution of active internal ribozyme between the nucleus and the cytoplasm. (Ren *et al.*, 1998; Crone et al., 1999)

25 An aspect of the invention is a procedure which allows selection of efficient cleavage sites in target RNAs using a library of random sequences. This library selection technique, derived from a procedure described by Lieber and Strauss, was used to select optimal target sites for the *HnudC* ribozymes. In brief, a single stranded DNA library (ssDNA) was created with a 5' region of 16 fixed nucleotides containing the T7

-17-

promoter, a region of 6 random nucleotides, a -GA-, another region of 9 random nucleotides, and another distinct region of 15 fixed nucleotides at the 3' end (16-N₆-GAN-N₉-15). This ssDNA pool was used to construct a double stranded DNA library (DSDNA) by PCR. The dsDNA was transcribed to make the RNA library. Target
5 *HnudC* RNA was made by *in vitro* transcription using *HnudC* cDNA as the template. An aliquot of the library RNA which contains approximately 1 x 10⁹ different random sequences was added. After 20 minutes incubation at 37°C, the reaction was separated in 8% polyacrylamide gel under non-denaturing conditions. Bound library RNAs were recovered with the target RNA and then converted to DNA by RT-PCR. This double
10 stranded DNA was transcribed into RNA again by T7 RNA polymerase. This procedure constitutes a "round" of selection.

This procedure was then repeated with the selected RNA library, and target RNA until the binding of library to target RNA no longer increased. Six rounds were generally sufficient. The selected transcripts, which are efficient in binding *HnudC* RNA, were
15 amplified by PCR, cloned, and 60 clones were sequenced. Six consensus potential high-affinity binding sites for the *HnudC* ribozyme were identified which contained a -GA- internal site. Oligonucleotides containing the fixed 5' and 3' nucleotide sequences described herein and the specific identified oligonucleotide sequences were synthesized to make an internal ribozyme and then tested. Individually and in combination, they were
20 able to efficiently cleave *HnudC* mRNA *in vitro* (FIG. 9).

MATERIALS AND METHODS

Isolation and Sequence Analysis of the Human *NudC* Gene.

To clone the human *nudC* gene, an EST human cDNA Database (TIGR) was searched and identified two cDNA clones with a high degree of homology to *A. nidulans*
25 *nudC*. These two cDNA clones, obtained from the ATCC (108447, from skin, and 108449, from ovary) were inserted into a pBluescript SK-vector in *Escherichia coli* hosts. DNA was prepared from these plasmids with a standard large scale plasmid preparation and cesium purification of DNA. The DNA was sequenced using Applied Biosystems Taq Dyedeoxy Terminator Cycle sequencing kit as described by the manufacturer. (Pu
30 *et al.*, 1995). Neither cDNA clone contained the 5' end of the *HnudC* gene. The cDNA from skin (0.9 kb) was random primer labeled and used to probe a human Multi-tissue Northern Blot purchased from Clontech (Palo Alto, CA). The β-actin cDNA probe

-18-

supplied with the Multi-tissue Northern Blot (Clontech) was also random primer labeled and used to probe the same blot. Heart muscle demonstrated significant expression of *HnudC* mRNA. Marathon-Ready Human Heart cDNA was obtained from Clontech and used to perform RACE and 3' RACE (Clontech) using synthesized primers selected from the partial *HnudC* sequence. The 5' RACE primer was 5'-TTCTGTTCGTCTGAAGTTGGCAGC-3' and the 3' RACE primer was 5'-CAATGAAGTGAAGGTGGAGGAGAG-3'. Single 5' and 3' RACE PCR products were obtained. A Southern blot was prepared with the RACE PCR products and probed with random primer labeled partial human *nudC* cDNA from the skin clone. Single 5' and 3' products were confirmed and the 5' and 3' RACE PCR products were sequenced.

Transformation and Complementation of the *A. nidulans nudC3* Mutant with Human *nudC* cDNA.

To functionally test human *nudC* in *A. nidulans* the portion of *HnudC* that is homologous was subcloned to *nudC* into the *A. nidulans* expression vector pAL515 using forward primer 5'-AAGGTACCAAGATGGACTCCCCAGGGAAGCAGGATACT-3' and reverse primer 5'-AAGGATCCAAGAAAGTTGGGTGGTTGCAGCTC-3' for the PCR. The PCR product was digested with BamHI and KpnI before ligation into pAL5. Three control plasmids were employed, the empty vector pAL5, pAL5 containing *nudC* cDNA and *nudC* cDNA cloned into pUC18.7 These plasmids were transformed into a *nudC3* containing strain (A01) selecting for *pyr4+* transformants at permissive temperature using standard techniques. Protoplasts of A01 were generated using cell wall degrading enzymes and plasmid DNA was introduced using polyethylene glycol and calcium. Transformed protoplasts were plated on osmotically balanced media in the absence of uridine to select for *pyr4+*. (Osmani *et al.*, 1987) For *nudC* cDNA cloned into pUC18 plasmid *pyrG* was co-transformed to supply the nutritional marker. (Oakly *et al.*, 1987) Random transformants were then tested for temperature sensitivity at 42°C on media non-inducing and non-repressing for the *alcA* promoter (minimal media glycerol).

Preparation of Antibodies to NUDC.

Antibodies were raised to synthesized peptides prepared to 1) a 12 amino acid sequence in the carboxy terminus of NUDC conserved in *A. nidulans*, *Drosophila*, rat, and human (GC M V E K M M Y D Q R Q K) or 2) a 15 amino acid sequence in the mid

-19-

region of human NUDC (N G S L D S P G K Q D T E E D). The peptides were initially coupled to activated BSA supercarrier using the Inject Activated Supercarrier System from Pierce (Rockford, Illinois). For rabbits prepared later, the peptide was coupled to KLH Sulfo-MBS (Pierce) and injected with Freund's adjuvant (first two injections) followed by Alum (Pierce). Antibodies were affinity purified with the immobilized peptide using the SulfoLink Kit (Pierce). To test recognition of *A. nidulans* NUDC by these antibodies, protein extracts from the A01 (*nudC3*, *wA2*, *nicA2*, *pabaA1*, *pyrG89*) derived *Aspergillus* strain 6A, containing *alcA* driven expression of *nudC*, were prepared with or without induction of *nudC* expression with ethanol and the protein content of extracts was quantitated. Protein was isolated in the presence of protease inhibitors by grinding frozen cells in a mortar and pestle. (Osmani *et al.*, 1990)

Preparation of BFU-E Derived Erythroblasts.

Peripheral blood was obtained from normal volunteer donors at The Milton S. Hershey Medical Center under protocols approved by the Institution's Clinical Investigation Committee. Peripheral blood mononuclear cells were separated on Ficoll-Paque (Pharmacia, LKB Biotechnology, Inc., Piscataway, NJ) and cultured on 0.9% methylcellulose media containing 30% fetal calf serum, 9.0 mg/ml deionized bovine serum albumin (Cohn fraction V; Sigma Chemical Co., St. Louis, MO), 1.4×10^{-4} mol/liter β -mercaptoethanol, and 2 U/ml erythropoietin (recombinant Epo > 100,000 U/mg; R & D Systems, Inc., Minneapolis, MN). Single BFU-E, when cultured in methylcellulose, proliferate and differentiate over 14 days to form large colonies containing $1-5 \times 10^4$ mature erythroblasts. These cells can be removed from culture at different days to study a well defined population of normal human cells at distinct stages of maturation. (Zhang *et al.*, 1997)

Cells were plated at 1 to 1.5×10^5 cells/mL, and cultures were incubated in humidified 4% CO₂ at 37°C. Erythroid colonies were counted and harvested at day 7, 10, or 14 of culture. One hundred to 1,000 (day 7) BFU-E-derived colonies were plucked and pooled on each day and the average number of erythroid cells per colony was determined.

Cytocentrifuged slides of BFU-E-derived erythroblasts removed from culture on days 7, 10 or 14 were prepared.

-20-

Day 7 cells are poorly hemoglobinized blasts with a large proliferative capacity, day 10 cells are partially hemoglobinized proerythroblasts and basophilic normoblasts with decreased proliferative capacity, and day 14 cells are terminally differentiating polychromatophilic and orthochromatic normoblasts. Cells from maturing BFU-E-derived colonies were plucked from culture on days 7, 10 and 14. Cytocentrifuge preparations of aliquots of BFU-E-derived cells routinely identified > 99% as erythroid precursors.

Hematopoietic Cell Lines.

TF-1 cells, a human erythroleukemia cell line, (Kitamura *et al.*, 1989) ATCC CRL-2003 were maintained in RPMI 1640 medium containing 10% fetal calf serum and 1-2 ng/ml human recombinant GM-CSF (R & D Systems, Minneapolis, MN). To examine *HnudC* induction, TF-1 cells were removed from growth factor for 24 hours and then stimulated with 2 ng/ml GM-CSF. Samples were collected at intervals over 0-24 hours. Acute lymphoblastic leukemia cell lines Jmi (ATCC CRL 10423 ATCC CRL 243), Reh (ATCC CRL 8286), AML-193 cells (ATCC CRL 9589, and K562 were obtained from the ATCC and cultured under recommended conditions.

Immunoblotting.

Whole cell lysates were prepared by suspending 1×10^6 BFU-E-derived cells or TF-1 cells in lysis buffer (50 mM Tris HCL, pH 8.0; 150 mM NaCl; 0.05% NP40; 0.8 mM PMSF; 0.01 mg/ml leupeptin; 0.01 mg/ml aprotinin; 100 mM NaF; 1 mM EDTA; and 1 mM EGTA). The protein content was measured and a known amount of protein was combined with 2X sample buffer and loaded onto each lane of a 10% polyacrylamide gel. Equivalent loading of all blots was confirmed by Acid Red 150 Ponceau SS (Sigma) staining.

Nuclear and cytoplasmic fractions were prepared as described by Schreiber. (1989) Cell pellets from $1-10 \times 10^6$ cells were resuspended in ice-cold lysis buffer (10 mM Hepes, pH 7.9; 10 mM KCl; 0.1 mM EDTA; 0.4% NP40; 1 mM DTT; 0.5 mM PMSF; and 1% volume of protease inhibitor cocktail). Cells were pipetted and then left to stand on ice for five minutes. The lysates were spun, and the supernatant was used for the cytosol preparation. The nuclear pellet was extracted with ice cold buffer (20 mM Hepes, pH 7.9; 400 mM NaCl; 1 mM EDTA; 1 mM DTT; 1 mM PMSF), spun, and the supernatant was used as the nuclear extract.

-21-

Proteins were electroblotted onto Hybond-ECL (nitrocellulose). Membranes were blocked with 5% BSA overnight at 4°C and 5% nonfat dry milk for one hour at room temperature in TTBS (20 mM Tris HCl, pH 7.5; 500 mM NaCl; 0.05% Tween-20). Membranes were incubated with anti-NUDC antibodies (immune sera diluted 1/200 for anti-CT antibody or 1/1000 for anti-MID, purified antisera 1/10) or anti-Iκβ,21 (diluted 1/2000) as a control for 3 hours at room temperature. Detection was with protein A - peroxidase (diluted 1/50,000) for NUDC or secondary antibody conjugate (diluted 1/2000) for anti- Iκβ, followed by ECL (Amersham Life Sciences, Buckinghamshire, England). Bands on autoradiography were quantitated with a Molecular Dynamics Densitometer using Quantity One Software from Protein-Data Bases Incorporated (Huntington, N.Y.).

RT-PCR Analysis of *NudC* mRNA in BFU-E Derived Cells.

Total RNA was isolated from 1×10^6 to 1×10^7 BFU-E derived cells or TF-1 cells by RNeasy™ Total RNA Kit (Qiagen, Chatsworth, CA). RT-RNA (cDNA) was made by Reverse Transcription System (Promega, Madison, WI) and PCR reaction was performed with the Perkin Elmer Gene AMP PCR Reagent Kit (Perkin Elmer, Roche Molecular Systems, Branchburg, NJ). A dose response curve and cycle number for each target gene were first determined to establish the optimal conditions for RT-PCR. (Zhang *et al.*, 1997)

RNA is required for RT-PCR detection. To construct the standard dose response curve, 0 to 1,000 ng of total cellular RNA from normal BFU-E derived cells at day 10 of culture was used to make 10 μL of RT reaction mixture. Two microliters of the cDNA was then amplified in 25 μL of PCR reaction mixture. Based on standard-dose response results, the linear range of cycle number was determined using a quantity of RNA on the slope of the dose-response curve, 2 μCi of (α - 32 P)deoxyadenosine 5'-triphosphate (daTP) was added to each PCR reaction for further kinetic analysis. After PCR, 10-μL aliquots were electrophoresed on 1.2% agarose gel and the amount of radioactivity incorporated into each band was measured by phosphoimager analysis.

In experiments, 20 ng of RT reaction of *HnudC* RT-RNA (cDNA) was amplified for 32 cycles (denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds and extension at 72°C for 45 seconds). 18S rRNA was similarly amplified for standardization. A small quantity (one fifth of RT reaction or 0.1 ng) of 18S rRNA RT-

-22-

RNA (cDNA) was amplified for 22 cycles (denaturation at 95°C for 30 seconds, annealing at 52°C for 30 seconds and extension at 72°C for 45 seconds). Equal amounts of Taq Start antibody (Clontech, CA), used to enhance specificity, were incubated with Taq DNA polymerase for 5 minutes before addition to PCR reaction mixture. The following 5' and 3' primers were used: *HnudC*: 5' primer,

5'-CAATGAAGTGAAGGTGGAGGAGAG-3'; 3' primer,

5'-TTCTGTTCGTCTGAAGTTGGCAGC-3'; 18S rRNA:

5' primer, 5'-GAAAGTCGGAGGTTCTGAAGA-3'; 3' primer,

5'-ACCAACTAAGAACGGCCATG-3'.

10 Northern Blot Analysis of RNA.

RNA was prepared from TF-1 cells at different time intervals following GM-CSF stimulation with the RNeasy™ Total RNA Kit (Qiagen). RNA samples (40 µg/lane) were separated on 1.2% agarose, formaldehyde gels and alkaline transferred to Zeta-Probe GT Genomic Tested Blotting Membrane (BioRad). Membranes were prehybridized for one hour at 37°C in 50% formamide, 5XSSC, 50 mM NaPO₄ pH 6.5, 0.5% milk, 1% SDS, 0.5 mg/ml salmon sperm DNA, 10% Dextran Sulfate. The *HnudC* probe was prepared from cesium purified ATCC 108447 plasmid cDNA, restriction digested with EcoRI and XhoI, followed by purification from Sea Plaque gel. The DNA was labeled in Sea Plaque with 50 µCi 32P dCTP by random primer labeling using DNA polymerase I Klenow enzyme fragment and random hexamers according to the directions of the manufacturer (Promega, Madison, WI). Hybridization was performed for 16 hours at 42°C by adding 2X10⁶ cpm/ml to hybridization buffer. Filters were washed at room temperature, followed by autoradiography.

20 Immunohistochemistry.

25 Bone marrow biopsies from normal donors were obtained under protocols approved by the Pennsylvania State University's Clinical Investigation Committee. BFU-E derived erythroblasts were removed from culture at day 7, 10 and 14 and cytocentrifuge preparations were prepared. Slides were fixed in acetone at 4°C for ten minutes, then stored at 4°C until used. Endogenous peroxidase was quenched with H₂O₂ and rabbit serum was used to block nonspecific binding. Slides were incubated with a 30 1:10 dilution of affinity purified anti-NUDC or preimmune antibodies at room temperature for 1 hour. A biotinylated secondary antibody from the Vectastain Rabbit

-23-

ABC kit (Vector Labs) was used according to the manufacturer's recommendations, with AEC as the chromogen. (Faleni and Taylor, 1983) The cells were counterstained with hematoxylin.

Antisense Oligonucleotide Studies

5 Antisense/sense phosphorothioate oligonucleotides were obtained from Genosys (Woodlands, Texas). Antisense (5'-AGCAACATGCCGTCGAACCGCTCC-3') and sense (5'-GGAGCGGTTTCGACGGCATGTTGCT-3') oligonucleotides targeted to the *HnudC* N-terminus were obtained. Optimal treatment time and oligonucleotide dose were determined by transfecting TF-1 cells for different time periods with different
10 concentrations of antisense/sense phosphorothioate oligonucleotides and lipofectin using the recommended procedures for transient transfection of suspension cells (Life Technologies). TF-1 cells were plated at $3-4 \times 10^5$ /ml in Opti-MEM I media (Gibco) with 2.5 or 5 ug/ml DNA and 10 ul lipofectin/ml. TF-1 cells were incubated with phosphorothioate oligonucleotides for 6 hours at 37° in a CO₂ incubator, then washed
15 and cultured in TF-1 growth media containing GM-CSF for 72 hours. Cells were then counted and harvested for Western blotting.

Preparation and Targeting of Ribozymes

A procedure was designed which allows selection of efficient cleavage sites in target RNAs using a library of random sequences. This library selection technique,
20 derived from a procedure described by Lieber and Strauss, was used here to select optimal target sites for the *HnudC* ribozymes. In brief, a single stranded DNA library (SSDNA) was created with a 5' region of 16 fixed nucleotides containing the T7 promoter, a region of 6 random nucleotides, a -GA-, another region of 9 random nucleotides, and another distinct region of 15 fixed nucleotides at the 3' end (16-N₆-
25 GAN-N₉-15). This SSDNA pool was used to construct a double stranded DNA library (DSDNA) by PCR. The DSDNA was transcribed to make the RNA library. Target *HnudC* RNA was made by *in vitro* transcription using *HnudC* cDNA as the template. An aliquot of the library RNA which contains approximately 1×10^9 different random sequences was added. After 20 minutes incubation at 37°C, the reaction was separated
30 in 8% polyacrylamide gel under non-denaturing conditions. Bound library RNAs were recovered with the target RNA and then converted to DNA by RT-PCR. This double stranded DNA was transcribed into RNA again by T7 RNA polymerase. This procedure

-24-

constitutes a round of selection. This was then repeated with the selected RNA library and target RNA until the binding of library to target RNA no longer increased. Six rounds were sufficient. The selected transcripts, which are efficient in binding *HnudC* RNA, were amplified by PCR, cloned, and 60 clones were sequenced. Six consensus potential high-affinity binding sites for the *HnudC* ribozyme were identified which contained a -GA- internal site. Oligonucleotides containing the fixed 5' and 3' nucleotide sequences described above and the specific identified oligonucleotide sequences were synthesized to make an internal ribozyme and then tested. Individually and in combination, they were able to efficiently cleave *HnudC* mRNA *in vitro*. In these experiments, conditions for cleavage of target RNA were not optimized since our main objective was to demonstrate cleavage and appropriately sized products. Internal ribozymes may be subcloned into the ribozyme cassette designated pClip at BglIII and MfeI sites to make *HnudC* triple ribozymes.

In conclusion, a new human gene involved in hematopoietic cell proliferation was identified and sequenced. The ability of human *nudC* to restore normal colony growth to an *A. nidulans* mutant, the inducible expression in response to hematopoietic growth factor stimulation, and the requirement for *nudC* in cell growth in antisense studies, all suggest *nudC* has an essential function in hematopoiesis, which is part of its conserved role from filamentous fungi to humans.

20

EXAMPLES

The following examples illustrate embodiments and aspects of the invention:

Example 1: Use of Antisense Molecules to Control HNUDC.

Functional studies were performed with TF-1 cells using phosphorothioate antisense or sense oligonucleotides targeted to the N-terminus of *HnudC* mRNA. Optimal treatment time and oligonucleotide dose were determined by transfecting TF-1 cells for different time periods with different concentrations of antisense/sense phosphorothioate oligonucleotide and lipofectin. A six hour treatment with lipofectin and 2.5 or 5 ug/ml antisense or sense oligonucleotide was optimal (FIG. 8A) since these conditions produced little growth inhibition with the sense phosphorothioate oligonucleotide compared to treatment with lipofectin alone. In contrast, antisense oligonucleotide to the same coding region as sense inhibited TF-1 proliferation to 33±6% (p≤0.001) of lipofectin control. A second antisense oligonucleotide inhibited TF-1

proliferation to $46 \pm 6\%$ of control ($p < 0.001$). This inhibition was dose-dependent and was observed in three experiments. However, nonspecific toxicity was observed with the sense oligonucleotide when concentrations were increased further.

To determine the effect of antisense/sense oligonucleotide treatment on NUDC
5 protein levels, immunoblot analysis was performed on lipofectin alone, sense or antisense treated TF-1 cells. Results are shown in FIG. 8B and are consistent with effects on TF-1 cell growth observed. NUDC protein was significantly reduced in TF-1 cells treated with 5 ug/ml antisense oligonucleotides compared to sense or lipofectin control.

An aspect of the invention is a human homolog of the *A. nidulans nudC* gene.
10 The human homolog was determined. In *A. nidulans*, *nudC* is of critical importance for normal colony growth through its essential role in nuclear migration. Extensive homology of human NUDC with rat, *Drosophila* and *A. nidulans* NUDC, particularly in the carboxy terminus, suggests that this is an important protein with a critical function conserved throughout much of evolution. Human *HnudC*, over the region conserved
15 between it and *nudC*, is capable of fulfilling the function of *A. nidulans nudC*. The high conservation of both primary sequence and function suggests that *nudC* is critical to both filamentous fungi and vertebrates.

A role for *HnudC* in hematopoietic proliferation was established using three different experimental systems. In the first, normal erythroid and myeloid human bone
20 marrow precursors demonstrated high levels of HNUDC, which dramatically declined in terminally differentiating cells. In the second, normal human progenitor-derived erythroblasts, expression of both *HnudC* mRNA and protein was highest during the proliferative stage of culture. As these cells exited the proliferative stage and approached terminal differentiation, the levels of both mRNA and protein declined. These effects
25 were specific, as a control protein remained constant in its level of expression and globin mRNA and protein have both previously been shown to increase in later differentiation. Third, expression of *HnudC* mRNA and protein was significantly enhanced after stimulation of proliferation of a human erythroleukemia cell line (TF-1) with GM-CSF. In addition, a far higher level of *HnudC* expression was observed in this erythroleukemia
30 cell line, which has a high proliferative capacity, compared to normal progenitor-derived erythroblasts, which have a more limited proliferative capacity. This high level of HNUDC expression was also observed in four other hematopoietic cell lines. In addition,

-26-

20-fold enhancement of HNUDC in lysates of bone marrows of patients with leukemia was observed. The ability of antisense *HnudC* oligonucleotides, but not sense, to inhibit the growth of TF-1 cells demonstrates the essential function of this protein in hematopoietic proliferation.

5 **Example 2: Subcellular Localization of HNUDC.**

Cellular localization of HNUDC was examined by immunofluorescent staining of TF-1 cells using specific affinity-purified antibodies to both the C-terminal and mid-regions of the protein. HNUDC was observed in both the nucleus and the cytoplasm with both antibodies but not with pre-immune sera or antibody incubated with cognate peptide
10 prior to cell staining, while no specific staining of cytoskeletal elements was observed in the cytoplasm.

HNUDC localized primarily in the paranuclear cytoplasmic region and is polarized to one side of the cell. This is consistent with immunofluorescence studies which demonstrated that RNUDC is a cytoplasmic protein localized to one side of the
15 nucleus in Nb2 T cells, COS-1 cells, and 2AG10 adenocarcinoma cells and that RNUDC was partly associated with the Golgi (Morris *et al.*, 1998, Lucocq *et al.*, 1989). RNUDC may play a role in the reorientation of the centrosome that occurs after antigen presentation to T cells. In addition to its localization to the paranuclear region in hematopoietic cells, a portion of HNUDC was also detected in discrete nuclear foci.

20 Nuclear staining consisted of discrete foci. Occasionally cells were observed which had little nuclear staining but contained bright foci in the cytoplasm surrounding the DNA. These cells were always in pairs, suggesting that they may be dividing cells and careful examination of cells doubly stained with HNUDC antibodies and tubulin confirmed that these cells were in late anaphase, telophase, or undergoing cytokineses.
25 This staining of cytoplasmic foci during late anaphase through to cytokinesis was observed with both antibodies and upon fixation in either cold methanol or para-formaldehyde. To determine which subnuclear compartment HNUDC was localized to, double immunofluorescent staining was carried out with the HNUDC antibodies and a panel of antibodies specific for known nuclear proteins. Interphase HNUDC nuclear foci
30 were found to colocalize with the SC-35 protein, which is a component of spliceosomes. SC-35 is an essential splicing factor which also colocalizes with interchromatin granules and perichromatin fibrils. Examination of cells in late anaphase, telophase, or undergoing

-27-

cytokinesis indicated that the HNUDC foci in these cells also colocalized with SC-35 during these stages of the cell cycle. Similar colocalization of the nuclear fraction of HNUDC with SC-35 was also observed in a rhabdomyosarcoma cell line. The presence of HNUDC in foci surrounding the DNA of dividing cells suggests that it may be playing a role in cell division/proliferation by controlling nuclear movements at cytokinesis.

Example 3: Preparation and Targeting of Ribozymes to *HnudC*

Inactivation of gene expression by ribozymes is a powerful technique for studying the function of a gene product and has therapeutic potential as well. Triple ribozymes (TRz) are used to inhibit *HnudC* mRNA expression. The basic reagent encompasses a TRz whose expression is controlled by an inducer or tissue-specific promoter. The TRz includes an internal trans-acting ribozyme, which can bind to target RNA. The internal ribozyme is flanked by 2 cis-acting ribozymes which function to cut themselves off, liberating the internal ribozyme. The liberated internal ribozyme is 3-8 times more active than the same ribozyme within non-specific flanking sequences, and the self-liberation process provides a distribution of active internal ribozyme, between the nucleus and the cytoplasm.

A procedure was designed which allows selection of efficient cleavage sites in target RNAs using a library of random sequences. This library selection technique, derived from a procedure described by Lieber and Strauss, was used here to select optimal target sites for the *HnudC* ribozymes. In brief, a single stranded DNA library (ssDNA) was created with a 5' region of 16 fixed nucleotides containing the T7 promoter, a region of 6 random nucleotides, a -GA-, another region of 9 random nucleotides, and another distinct region of 15 fixed nucleotides at the 3' end (16-N₆-GAN-N₉-15). This ssDNA pool was used to construct a double stranded DNA library (dsDNA) by PCR. The dsDNA was transcribed to make the RNA library. Target *HnudC* RNA was made by *in vitro* transcription using *HnudC* cDNA as the template. An aliquot of the library RNA which contains approximately 1 x 10⁹ different random sequences was added. After 20 minutes incubation at 37°C, the reaction was separated in 8% polyacrylamide gel under non-denaturing conditions. Bound library RNAs were recovered with the target RNA and then converted to DNA by RT-PCR. This double stranded DNA was transcribed into RNA again by T7 RNA polymerase. This procedure constitutes a "round" of selection. This was then repeated with the selected RNA library and target

-28-

RNA until the binding of library to target RNA no longer increased. Six rounds were sufficient. The selected transcripts, which are efficient in binding *HnudC* RNA, were amplified by PCR, cloned, and 60 clones were sequenced. Six consensus potential high-affinity binding sites for the *HnudC* ribozyme were identified which contained a -GA-
5 internal site. Oligonucleotides containing the fixed 5' and 3' nucleotide sequences described above and the specific identified oligonucleotide sequences were synthesized to make an internal ribozyme and then tested. Individually and in combination, they were able to efficiently cleave *HnudC* mRNA in vitro. In these experiments, conditions were optimized for cleavage of target RNA including ribozyme to target ratio or time of
10 incubation, since our main objective was to demonstrate cleavage and appropriately sized products. This procedure was employed with a number of other targets. With TRz targeted to hepatitis B virus mRNA, for example, 3 predominant ribozymes with catalytic activities 3200 X greater than that of a comparable ribozyme designed based upon mFold modeling of HBV RNAs were identified. In all cases, library selected ribozymes were
15 10^2 - 10^3 X more active than those designed based on mFold modeling. The internal ribozymes are identified herein all subcloning into the ribozyme cassette designated pClip at BglII and MfeI sites to make *HnudC* triple ribozymes. Self-liberation and target cleavage are tested as described, using P-labeled *HnudC* RNA transcribed *in vitro*.

Example 4: Diagnosis of a Cancer Using HNUDC

20 To diagnose a cancer, a sample of cells potentially harboring malignant cells is obtained, *e.g.* a bone marrow sample for leukemia, a piece of solid tumor tissue for breast cancer. Lysates are obtained from the candidate cells. Total protein is quantitated and the relative amount of HNUDC present is determined by antibody to HNUDC as disclosed herein using either a Western blot or ELISA. The value for HNUDC is
25 compared to a known quantity of standard. Standards are made by producing recombinant HNUDC in host strains, *e.g. E. coli* and expressing and measuring HNUDC by a BIORAD Protein Assay Kit. RNA will similarly be prepared from candidate cells and quantitation of HNUDC mRNA will be by RT-PCR.

Example 5: Staging of a Leukemia Using HNUDC (*HnudC*) - Expression

30 Following similar procedures as in Example 4, samples of HNUDC are obtained and compared to a standard obtained from samples of patients in clinically recognized

stages, *e.g.* “standard” and “high risk” in ALL leukemia, using standard statistical means to determine whether a patient’s HNUDC values fall within the range of a stage.

Example 6: Treatment of an Uncontrolled Cell Growth with Antisense to *HnudC*

- To treat individuals with an uncontrolled cell growth, antisense molecules,
- 5 ribozymes or other *HnudC* gene or gene products are delivered to a person to be treated. Delivery may be via liposomes or adenovirus, for example. Antisense molecules or ribozymes are preferably delivered in an expression vector, more preferably an inducer or tissue specific expression vector.

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-32-

CLAIMS

1. An isolated cDNA molecule that encodes a nuclear migration protein in human cells and is capable of complementing the *nudC* mutation of *A. nidulans*.
2. The cDNA molecule of claim 1, comprising the nucleotide sequence:

5

1 CTAGAGTGCA GAGCTCCGGG ACGTGGATCG GAGCCGGCGC
 GATGGGCGGA GAGCAGGAGG
 61 AGGAGCGGTT CGACGGCATG TTGCTGGCCA TGGCTCAGCA
 GCACGAGGGC GCGTGCAGG
 10 121 AGCTTGTGAA CACCTTCTTC AGCTTCCTTC GACGCAAAC
 AGACTTTTTC ATTGGAGGAG
 181 AAGAAGGGAT GGCAGAGAAG CTTATCACAC AGACTTTCAG
 CCACCACAAT CAGCTGGCAC
 241 AGAAGACCCG GCGGGAGAAG AGAGCCCGGC AGGAGGCCGA
 15 GCGGCGGGAG AAGGCGGAGC
 301 GGGCGGCCAG ACTGGCCAAG GAAGCCAAGT CAGAGACCTC
 AGGGCCCCAG ATCAAGGAGC
 361 TAACTGATGA AGAGGCAGAG AGGCTGCAGC TAGAGATTGA
 CCAGAAAAAG GATGCAGAGA
 20 421 ATCATGAGGC CCAGCTCAAG AACGGCAGCC TTGACTCCCC
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 481 AAGATGAGGA GGAAGATGAG AAGGACAAAG GAAAAGTAA
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 541 CAGACCTGCC CAATTACCGC TGGACCCAGA CCCTGTCGGA
 25 GCTGGACCTG GCGGTCCCTT
 601 TCTGTGTGAA CTTCCGGCTG AAAGGGAAGG ACATGGTGGT
 GGACATCCAG CGGCGGCACC
 661 TCCGGGTGGG GCTCAAGGGG CAGCCAGCGA TCATTGATGG
 GGAGCTCTAC AATGAAGTGA
 30 721 AGGTGGAGGA GAGCTCGTGG CTCATTGAGG ACGGCAAGGT
 GGTGACTGTG CATCTGGAGA

-33-

- 781 AGATCAATAA GATGGAGTGG TGGAGCCGCT TGGTGTCCAG
TGACCCTGAG ATCAACACCA
- 841 AGAAGATTAA CCCTGAGAAT TCCAAGCTGT CAGACCTGGA
CAGTGAGACT CGCAGCATGG
- 5 901 TGGAAAAGAT GATGTATGAC CAGCGACAGA AGTCCATGGG
GCTGCCAACT TCAGACGAAC
- 961 AGAAGAAACA GGAGATTCTG AAGAAGTTCA TGGATCAACA
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- 1021 AGGCTAAATT CAACTAGCCC CTGTTTTTTC CTCCCTGAAC
10 TCTTGGGGCT GAGCTGCAAC
- 1081 CACCCAACTT TCTTTCCAC TCTTCTCTGG GACTTGTGGG
CCTCAGGGCT TGGGGCAGGC
- 1141 ATGGGACTGG CCCAGGCACA CAGGTCCCGG GGCATCAGGA
GAAAGGCTGG GTCTTGGGAC
- 15 1201 CTTGTCCTCC CCAGTTGGCC TACTGTTACA CATTAAAACG
ATTTGCCAG CTCAAAAAAA
- 1261 AAAAAAAAAA AAAAAAAAAA A
3. Use of an antisense molecule complementary to a human nuclear migration gene to inhibit expression of the gene in malignant cells in humans.
- 20 4. The use of an antisense molecule of claim 3, wherein the malignant cells are bone marrow-derived cells from persons with acute lymphoblastic or myelogenous leukemia.
5. The use of the antisense molecule of claim 3, wherein the human nuclear migration gene is symbolized *HnudC*.
- 25 6. The use of the antisense molecule of claim 3, wherein the antisense molecule is a phosphorothioate oligonucleotide to *HnudC* mRNA.
7. Use of a labeled DNA or RNA probe capable of hybridizing to at least a portion of a human nuclear migration gene from a sample of a patient with a disease, to detect increased expression of the gene which would indicate the presence of an aggressive disease requiring intense therapy.
- 30

-34-

8. Use of ribozymes to inhibit the effects of a human nuclear migration gene on human cell proliferation by modulating production of HNUDC through interference with the mRNA produced by the gene.
9. The use of ribozymes of claim 8, wherein the human nuclear migration gene is *HnudC*.
10. Use of antibody directed to HNUDC quantitate HNUDC protein levels in malignant cells.
11. The use of claim 10, wherein malignant cells are selected from the group consisting of acute lymphoblastic and myelogenous leukemia cells.
- 10 12. The use of claim 10, wherein after quantitating the HNUDC protein levels, the levels are compared to standards to determine the clinical stage of the malignancy.
13. An expression vector comprising at least a portion of a human nuclear migration gene, and a suitable promoter.
- 15 14. The vector of claim 13, wherein the suitable promoter is a tissue specific promoter.
15. The vector of claim 12, wherein the expression is inducible.
16. An antibody to a fragment of a conserved sequence of the NUDC protein.
17. The antibody of claim 16, wherein the conserved amino acid sequence is MVEKMMYDOROK.
- 20 18. Use of an antibody to human NUDC to monitor expression of the NUDC protein in human cells.
19. Use of an antibody to human NUDC to detect patients with leukemia.
20. Use of an antibody to human NUDC in bone marrow to differentiate high risk from standard ALL patients.
- 25 21. An inhibitor of the DNA molecule as claimed in any one of claims 1 or 2, for use as a pharmaceutical.

Fig. 1

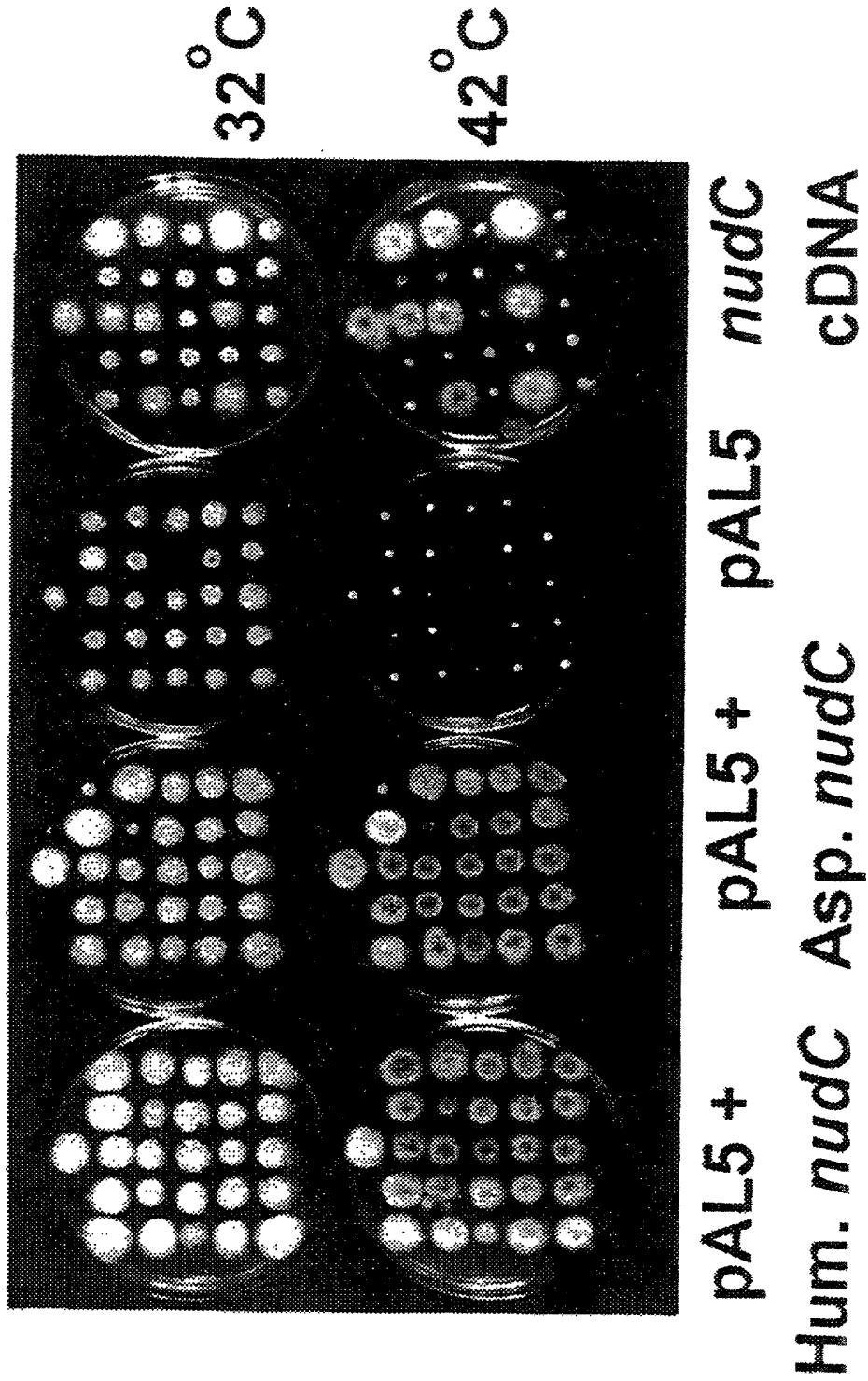
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RAT MGGEQEEERFDGMLLAMAQQHEGGVQELVNTFFFLRRKTDFFIGGEGMAEKLITQTFNHHNQLAQKARREKRARQETERREKAERAAR 90

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ASP MSEQEPSSADLAAREAEKQK-----AAEEAEQATLP-YKWTQTIR 41
CON S P D E EE A LP Y WTQT

HUM ELDLAVPFCVNFRLKGDVVDIQRRLRVGLKGQ--PAIIDGELYNEVKVEESSWLIED-----GKVVTVHLEKINKMEWWSRLVSS 260
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CON D V LKG D V V K IDG K ESSW E GK V HL K N MENW V

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RAT DPEINTKINPENSKLSDLDSETRSMVEKMMYDQRKSMGLPTSDEQKQKQEIILKFMQHPMEMDFS KAKFN 332
ASP APKIDVSKITPENSSLSLDLGETRAMVEKMMYDQRKEMGAPTSDEQRKMDILKFKQKEHPMEMDFS NAKIG 202
CON P I KI PENS LSDLD ETR MVEKMMYDOROK MG PTDSEQ K ILKKE HPMEMDFS AK

Fig. 2



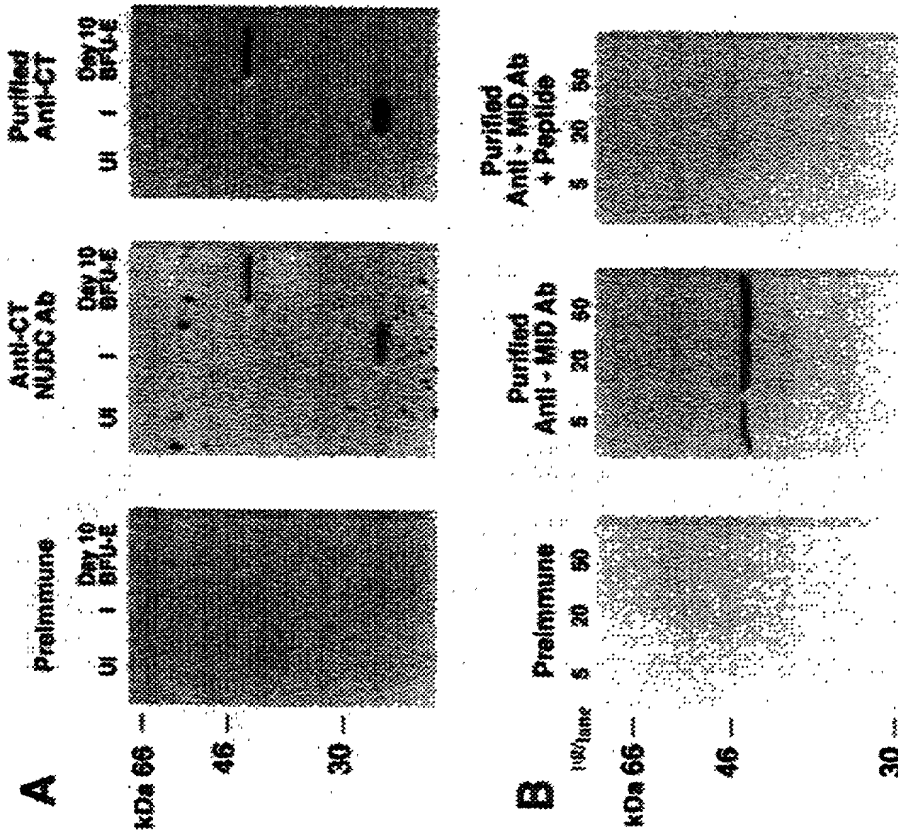


Fig. 3

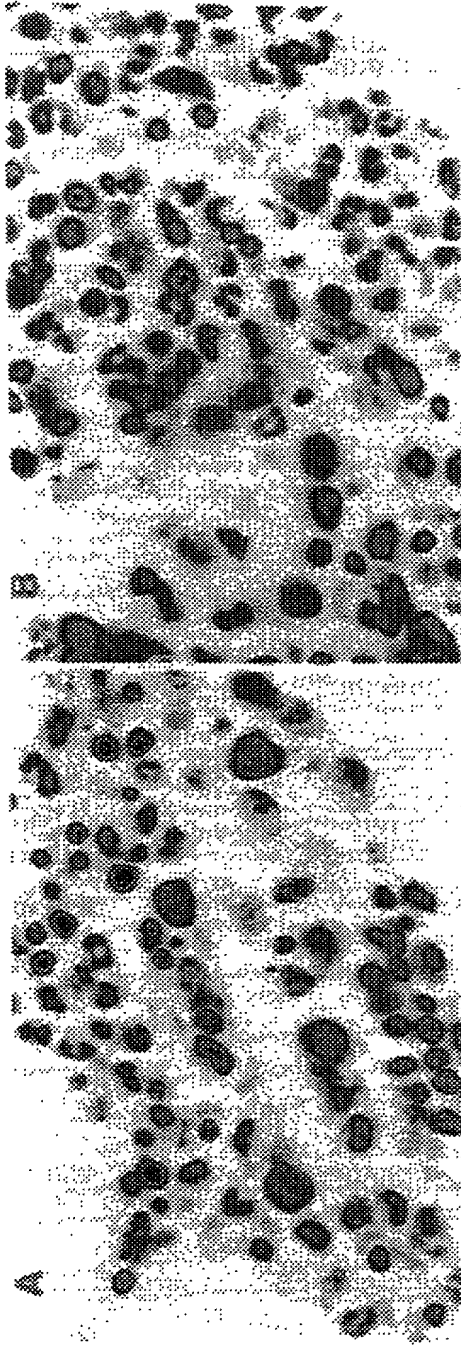


Fig. 4

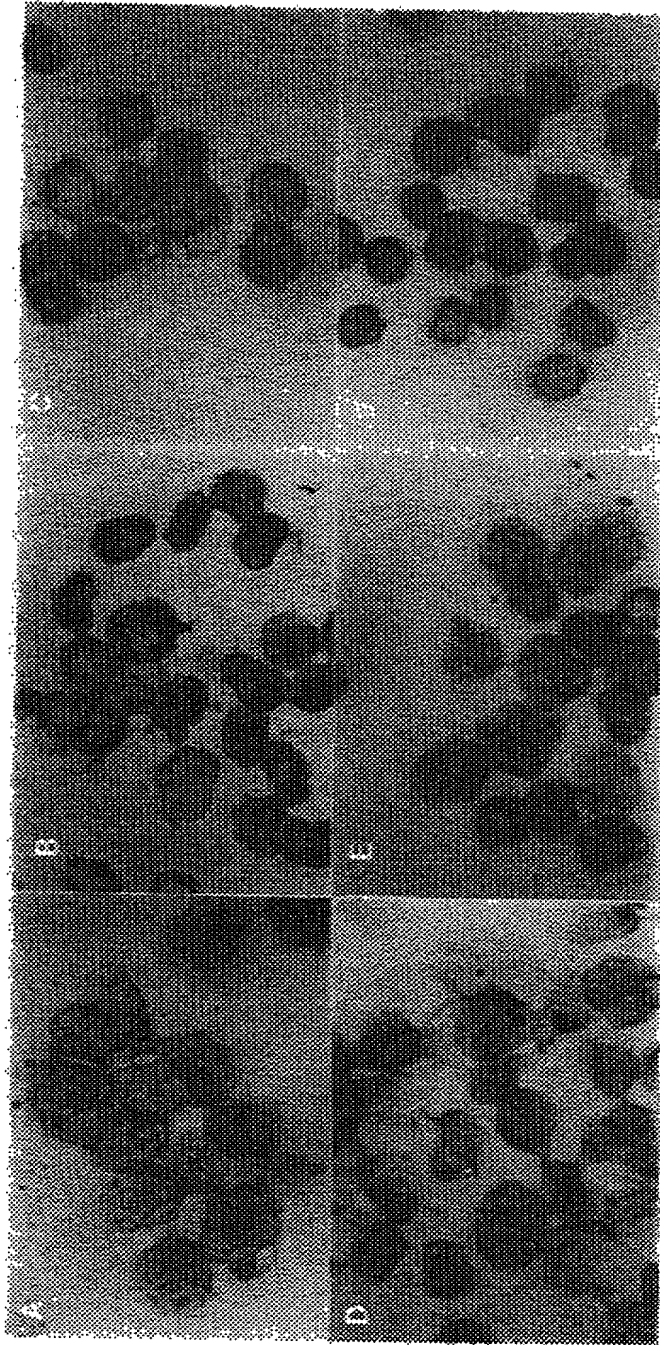
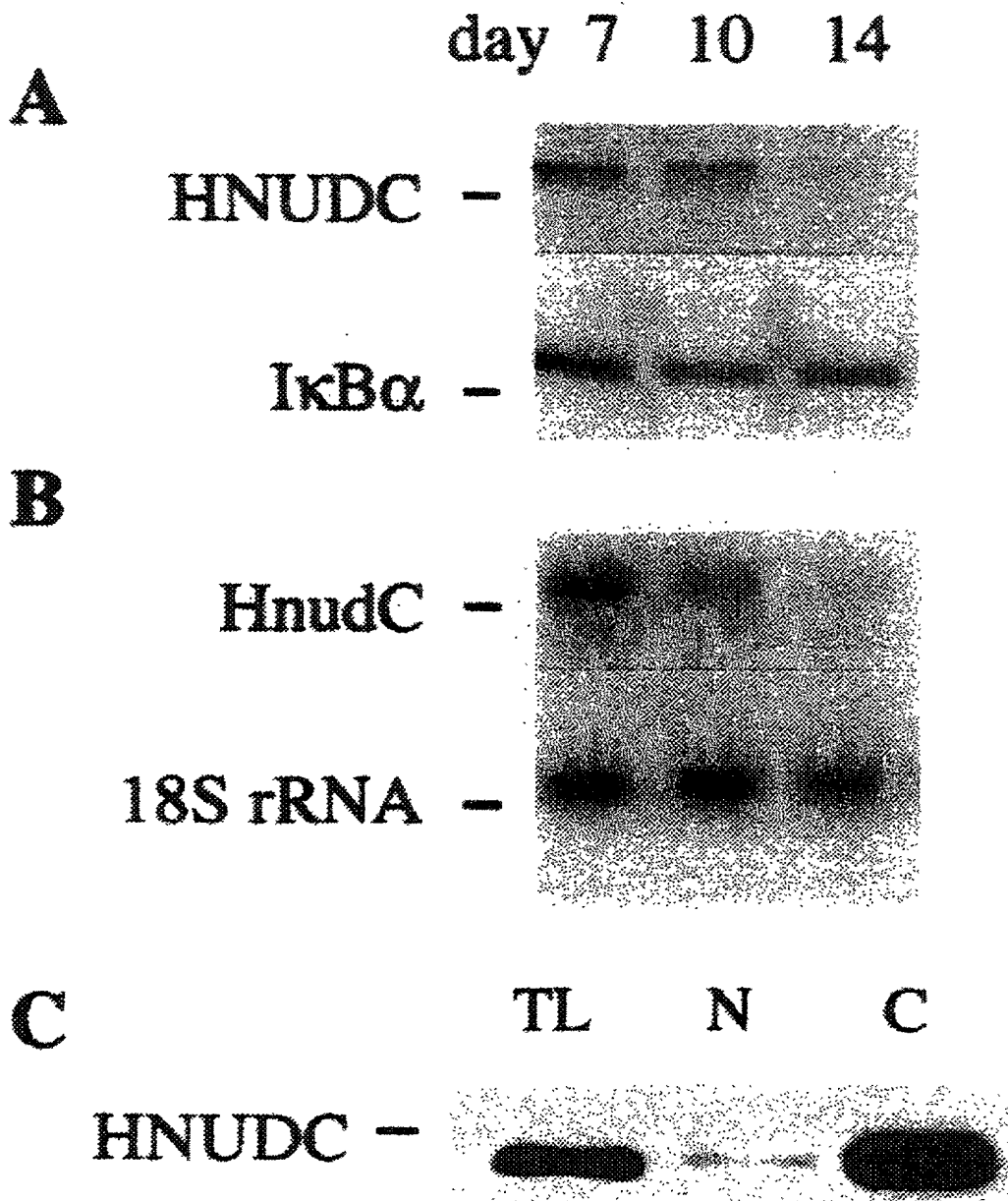


Fig. 5

Fig. 6



7/12

Fig. 7

Hr. 0 0.5 2 4 8 10 16 20 24

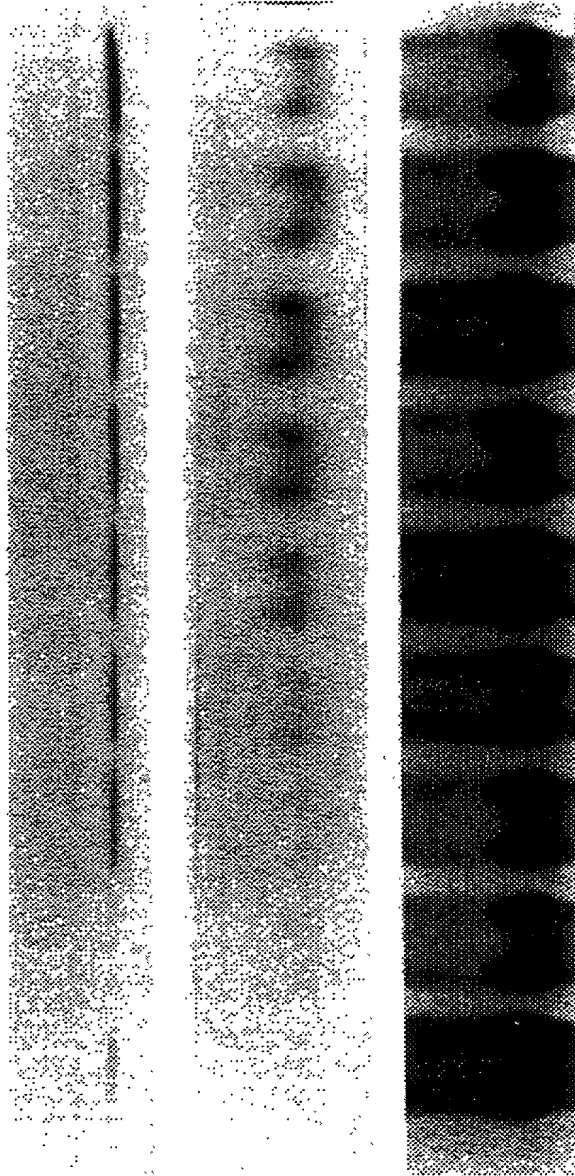
A

HNUDC

B

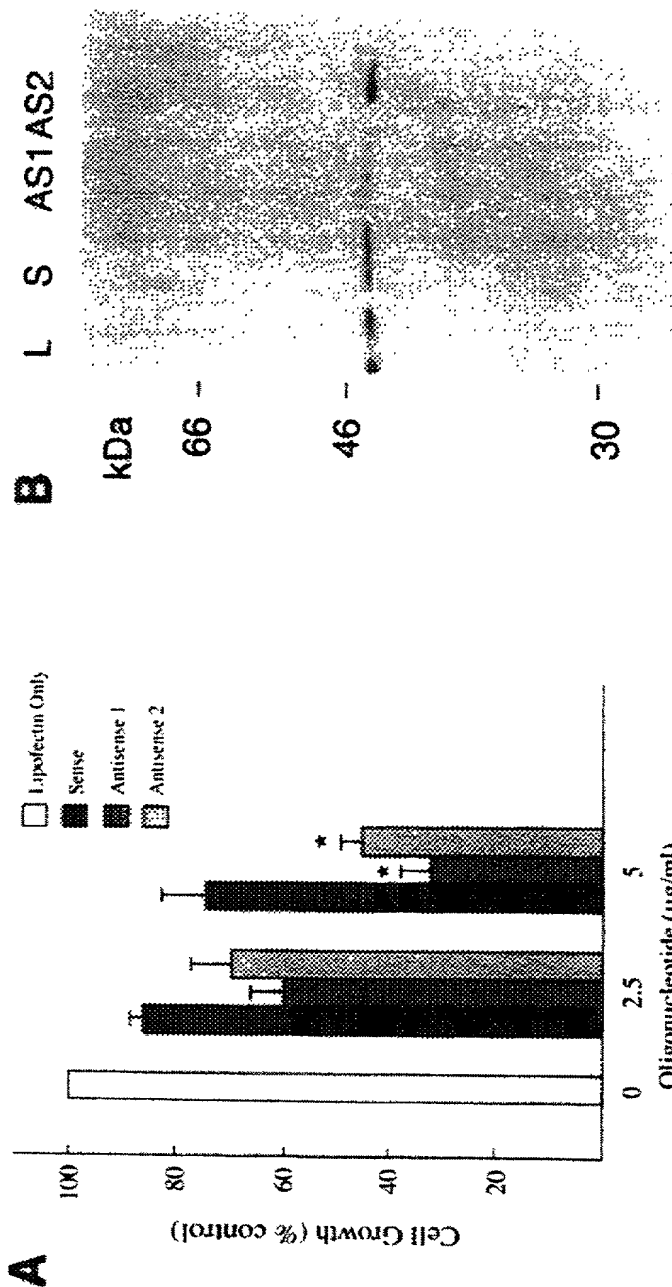
nudC

18S rRNA



8/12

Fig. 8



9/12

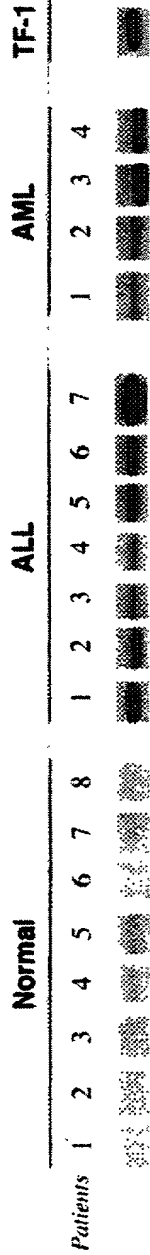


Fig. 9

10/12

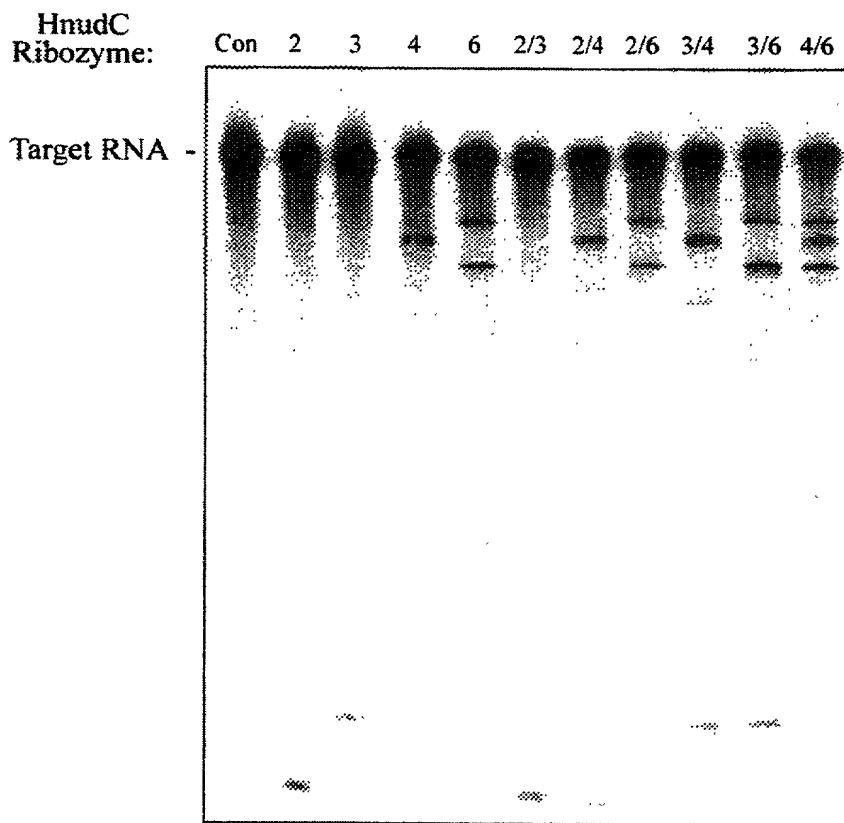


Fig. 10

11/12

FIG.11

1 CTAGAGTGCA GAGCTCCGGG ACGTGGATCG GAGCCGGCGC
GATGGGCGGA GAGCAGGAGG

61 AGGAGCGGTT CGACGGCATG TTGCTGGCCA TGGCTCAGCA
GCACGAGGGC GGCCTGCAGG

121 AGCTTGTGAA CACCTTCTTC AGCTTCCTTC GACGCAAAC
AGACTTTTTTC ATTGGAGGAG

181 AAGAAGGGAT GGCAGAGAAG CTTATCACAC AGACTTTCAG
CCACCACAAT CAGCTGGCAC

241 AGAAGACCCG GCGGGAGAAG AGAGCCCGGC AGGAGGCCGA
GCGGCGGGAG AAGGCGGAGC

301 GGGCGGCCAG ACTGGCCAAG GAAGCCAAGT CAGAGACCTC
AGGGCCCCAG ATCAAGGAGC

361 TAACTGATGA AGAGGCAGAG AGGCTGCAGC TAGAGATTGA
CCAGAAAAAG GATGCAGAGA

421 ATCATGAGGC CCAGCTCAAG AACGGCAGCC TTGACTCCCC
AGGGAAGCAG GATACTGAGG

481 AAGATGAGGA GGAAGATGAG AAGGACAAAG GAAAAGTAA
GCCCAACCTA GGCAACGGGG

541 CAGACCTGCC CAATTACCGC TGGACCCAGA CCCTGTCGGA
GCTGGACCTG GCGGTCCCTT

601 TCTGTGTGAA CTCCGGCTG AAAGGGAAGG ACATGGTGGT
GGACATCCAG CGGCGGCACC

661 TCCGGGTGGG GCTCAAGGGG CAGCCAGCGA TCATTGATGG
GGAGCTCTAC AATGAAGTGA

721 AGGTGGAGGA GAGCTCGTGG CTCATTGAGG ACGGCAAGGT
GGTACTGTG CATCTGGAGA

781 AGATCAATAA GATGGAGTGG TGGAGCCGCT TGGTGTCCAG
TGACCCTGAG ATCAACACCA

12/12

841 AGAAGATTAA CCCTGAGAAT TCCAAGCTGT CAGACCTGGA
CAGTGAGACT CGCAGCATGG

901 TGGAAAAGAT GATGTATGAC CAGCGACAGA AGTCCATGGG
GCTGCCAACT TCAGACGAAC

961 AGAAGAAACA GGAGATTCTG AAGAAGTTCA TGGATCAACA
TCCGGAGATG GATTTTTCCA

1021 AGGCTAAATT CAACTAGCCC CTGTTTTTTC CTCCTGAAC
TCTTGGGGCT GAGCTGCAAC

1081 CACCCAATT TCTTTCCAC TCTTCTCTGG GACTTGTGGG
CCTCAGGGCT TGGGGCAGGC

1141 ATGGGACTGG CCCAGGCACA CAGGTCCCGG GGCATCAGGA
GAAAGGCTGG GTCTTGGGAC

1201 CTTGTCCTCC CCAGTTGGCC TACTGTTACA CATTAAAACG
ATTTGCCCAG CTCAAAAAAA

1261 AAAAAAAAAA AAAAAAAAAA A

DECLARATION FOR PATENT APPLICATION

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

“USE OF A HUMAN HOMOLOG OF A NUCLEAR MIGRATION GENE FOR TREATMENT AND DIAGNOSIS OF CANCER”

the specification of which

(check one) [XXXX] is attached hereto.
[] was filed on _____ as United States Application Number _____ or PCT International Application Number _____ and was amended on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

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Prior Foreign Application(s)	<u>Priority Not Claimed</u>
_____	[]
(Number) (Country) (Day/Month/Year Filed)	
_____	[]
(Number) (Country) (Day/Month/Year Filed)	

I hereby claim the benefit under 35 U.S.C. § 119(e) of any United States provisional application(s) listed below.

_____	_____
(Application Number)	(Filing Date)
_____	_____
(Application Number)	(Filing Date)

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<u>PCT/US99/04996</u>	<u>05.03.99</u>	<u>Nationalized</u>
(Application Number)	(Filing Date)	(Status-patented, pending, abandoned)
_____	_____	_____
(Application Number)	(Filing Date)	(Status-patented, pending, abandoned)

DECLARATION FOR PATENT APPLICATION

I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

2

Thomas J. Monahan (Reg. No. 29,835); Janet E. Reed (Reg. No. 36,252)

Address all telephone calls to Thomas J. Monahan at telephone no. 814-865-6279.

Address all correspondence to Intellectual Property Office
The Pennsylvania State University
113 Technology Center
University Park, PA 16802

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full name of sole or first inventor (given name, family name): BARBARA MILLER

Inventor's signature: Barbara Miller Date: 8/15/00
Residence: Lewisburg, PA PA Citizenship: U.S.
Post Office Address: 101 South 4th Street, Lewisburg, PA 17837

Full name of second joint inventor, if any (given name, family name): STEPHEN OSMANI

Second Inventor's signature: Stephen A. Osmani Date: 08/17/2000 08/17/00
Residence: Danville, PA PA Citizenship: U.K.
Post Office Address: 5 Sunset Drive, Danville, PA 17821

Full name of third joint inventor, if any (given name, family name): GARY CLAWSON

Third Inventor's signature: Gary Clawson Date: 8/17/00
Residence: Bethesda, MD Citizenship: U.S.
Post Office Address: 5709 Rockmere Drive, Bethesda, MD 20816

Full name of fourth joint inventor, if any (given name, family name): MIN-YING ZHANG

Fourth Inventor's signature: Min-ying Zhang Date: 8/15/00
Residence: Hershey, PA Citizenship: China
Post Office Address: 120 Jacob's Creek Drive, Hershey, PA 17033

Full name of fifth joint inventor, if any (given name, family name): JAMES NORRIS

Fifth Inventor's signature: Date:
Residence: Citizenship:
Post Office Address:

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Inventor's signature: _____ Date: _____
Residence: _____ Citizenship: _____
Post Office Address: _____

Full name of second joint inventor, if any (given name, family name): STEPHEN OSMANI

Second Inventor's signature: _____ Date: _____
Residence: _____ Citizenship: _____
Post Office Address: _____

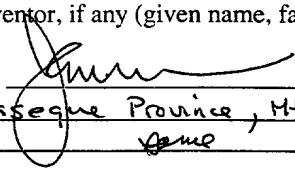
Full name of third joint inventor, if any (given name, family name): GARY CLAWSON

Third Inventor's signature: _____ Date: _____
Residence: _____ Citizenship: _____
Post Office Address: _____

Full name of fourth joint inventor, if any (given name, family name): MIN-YING ZHANG

Fourth Inventor's signature: _____ Date: _____
Residence: _____ Citizenship: _____
Post Office Address: _____

Full name of fifth joint inventor, if any (given name, family name): JAMES NORRIS

Fifth Inventor's signature:  Date: 8/11/2008
Residence: 1010 Casseque Province, Mt. Pleasant SC Citizenship: USA
Post Office Address: James

Vertical text on the left margin, possibly a scanning artifact or reference number.

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(check one) is attached hereto.
 was filed on 8/31/00 as United States Application Number 09/623,568
or PCT International Application Number _____ and was amended on
_____ (if applicable).

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Full name of sole or first inventor (given name, family name):

1-00
BARBARA MILLER

Inventor's signature: Barbara A. Miller US

Date: 2/22/01

Residence: Lewisburg, Pennsylvania

Citizenship: U.S.

Post Office Address: 101 South 4th Street, Lewisburg, PA 17837

PT

Full name of second joint inventor, if any (given name, family name): STEPHEN OSMANI

Second Inventor's signature: _____

Date: _____

Residence: Danville, Pennsylvania

Citizenship: U.K.

Post Office Address: 5 Sunset Drive, Danville, PA 17821

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Inventor's signature: _____ Date: _____
Residence: Lewisburg, Pennsylvania Citizenship: U.S.
Post Office Address: 101 South 4th Street, Lewisburg, PA 17837

2-00

Full name of second joint inventor, if any (given name, family name): STEPHEN OSMANT

Second Inventor's signature: Stephen A. Osman Date: 3/23/01
Residence: Columbus, Ohio Citizenship: U.K.
Post Office Address: 7915 Crossover Blvd, Columbus, OH 43235 *OH*

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